

Overexpression of α -acetolactate decarboxylase and acetoin reductase/2,3-butanediol dehydrogenase in *Arabidopsis thaliana*

by

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Declaration

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Abstract

Certain rhizobacteria have been identified as plant growth promoting rhizobacteria (PGPR), but the mechanisms involved and the exact mechanisms via which they operate still need to be fully elucidated. These bacteria live in symbiosis with the plants, by colonizing the roots or even entering the plant cells as endophytes. Once a symbiosis is established, certain beneficial substances are released to the plant including, but not limited to, volatile organic compounds (VOCs). Two such VOCs, acetoin and 2,3-butanediol have been shown to enhance general plant growth and initiate an induced systemic resistance (ISR) response.

In this study the genes responsible for the production of acetoin and 2,3-butanediol, α -acetolactate decarboxylase (*ALDC*) and acetoin reductase/2,3-butanediol dehydrogenase (*BDH1*) respectively, were isolated from *Aspergillus niger* ATCC 1015 and *Saccharomyces cerevisiae* W303. The acetoin precursor, acetolactate, is located in the chloroplast, thus the fully sequenced genes were cloned into plant expression vectors (pCambia2300 and pCambia1300) containing a ferredoxin-NADP⁺ reductase (FNR) transit peptide sequence for chloroplastic targeting. The genes were transformed into *Arabidopsis thaliana* Col-0 using an *Agrobacterium*-mediated floral dip method.

Transformed plants were tested for gene insertion and expression, and some of the lines were found to have undergone transgene silencing in the T₃ generation. Before growth promotion analysis between transgenic plants and untransformed control plants could commence, transformed double transgenic T₂ generation and single transgenic T₃ generation plants were tested for gene insertion and expression. The transgenic *ALDC* lines and one of the double transgenic lines showed some promise as they were significantly bigger than untransformed control plants in a number of physiological parameters, including leaf area, fresh and dry mass. Varying results were observed when wild type plants were tested against synthetic acetoin and 2,3-butanediol under short and long day lengths. The physical presence of acetoin and 2,3-butanediol in the transgenic lines was tested by means of enzyme assays, gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) analysis. The enzyme assays could not be utilized in the plant system tested, however, as identical trends in reduced nicotinamide adenine dinucleotide (NADH) oxidation were observed between transgenic and control plants. No detectable levels of acetoin could be identified by GC-MS or HPLC methods.

In general this study laid out the ground work for the incorporation of the *ALDC* and *BDH1* genes in *Arabidopsis*, with some preliminary growth comparison studies showing promise in the single *ALDC* and double *ALDC/BDH1* transgenic lines. A suitable detection method for acetoin and 2,3-butanediol still needs to be established for future studies.

Samevatting

Sekere risobakterieë is geïdentifiseer as plantgroeibevorderende risobakterieë, alhoewel die meganismes wat betrokke is by die bevorderende eienskappe nog nie ten volle verstaanbaar is nie. Hierdie bakterieë lewe in simbiose met plante deur die wortels te koloniseer of selfs om die plant selle te infiltrateer as endofiete. Sodra 'n simbiose gevestig is kan voordelige stowwe vir die plant vrygestel word, bv. vlugtige organiese verbindings. Acetoin en 2,3-butaandiol is twee sulke vlugtige organiese verbindings wat voorheen bewys is om plant groei te bevorder en om 'n geïnduseerde sistemiese weerstand reaksie te inisieër.

In hierdie studie is die gene verantwoordelik vir die vervaardiging van acetoin en 2,3-butaandiol, α -acetolactate decarboxylase (*ALDC*) and acetoin reductase/2,3-butanediol dehydrogenase (*BDH1*) onderskeidelik geïsoleer vanaf *Aspergillus niger* ATCC 1015 and *Saccharomyces cerevisiae* W303. Die acetoin voorloper, acetolactate, is geleë binne die chloroplast, daarom was die volledige volgorde bepaalde gene geklooneer binne-in plant uitdrukkings vektore (pCambia2300 and pCambia1300) bevattend 'n ferredoxin-NADP⁺ reductase (FNR) transito peptied volgorde vir chloroplast fokus. Die gene was in *Arabidopsis thaliana* Col-0 in getransformeer, deur gebruik te maak van 'n *Agrobacterium*-bemiddelde blom dompel metode.

Getransformeerde plante was getoets vir geen invoeging en uitdrukking. Sekere van die lyne was onderhewig aan transgeen onderdrukking in die T₃ generasie. Voor groei bevordering analiese tussen transgeiese plante en die kontroles uitgevoer kon word, was geen invoeging en uitdrukking voor-af getoets op dubbel getransformeerde T₂ generasie en enkel getransformeerde T₃ generasie plante. Die transgeniese *ALDC* lyne en een van die dubbel lyne het potensiaal getoon aangesien hulle aansienlik groter as die kontroles gegroei het in terme van blaar area, vars en droë massa. Wisselende resultate was ondervind vir kort en lang dae toe wilde tipe kontrole plante getoets was teen die sintetiese acetoin en 2,3-butaandiol. Die fisiese teenwoordigheid van acetoin en 2,3-butaandiol was voor getoets deur ensiem toetse, gas chromatography-mass spectrometry (GC-MS) en high-performance liquid chromatography (HPLC) analises in die transgeniese plante. Die ensiem toetse was onvanpas aangesien die gereduseerde nicotinamide adenine dinucleotide (NADH) oksidasie teen soortgelyke wyses vir kontrole en transgeniese plante plaasgevind het. Geen waarneembare vlakke van acetoin kon deur middel van die HPLC of GC-MS metodes geïdentifiseer word nie.

As alles saam gevat word, het hierdie studie die begin blokke van die invoeging van *ALDC* en *BDH1* in *Arabidopsis* neergelê. Voorlopige groei studies wys dat die enkel *ALDC* en die dubbel *ALDC/BDH1* transgeniese lyne belowende resultate bied. 'n Gepasde deteksie metode moet nog gestig word vir acetoin en 2,3-butaandiol vir toekomstige studies.

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Abbreviations

°C	Degrees Celsius
µg	Microgram
µm	Micrometer
µg/mL	Microgram per milliliter
µL	Microliter
µmoles	Micromoles
A	Absorbance
ACC	1-Aminocyclopropane-1-carboxylic acid
AHAS	Acetohydroxyacid synthase
ALDC	α-Acetolactate decarboxylase
ALS	Acetolactate synthase
AspGD	<i>Aspergillus</i> Genome Database
ATCC	American Type Culture Collection
BCAA	Branched-chained amino acids
BDH1	Acetoin reductase/2,3-butanediol dehydrogenase
bp	Base pair
ca.	Approximately
CAF	Central Analytical Facility
CaMV	Cauliflower mosaic virus
cDNA	Complementary deoxyribonucleic acid
cm	Centimeter
CTAB	Cetyltrimethylammonium
d	Day
DAD	Diode array detector
DCM	Dichloromethane
ddH ₂ O	De-ionised distilled water
dH ₂ O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol

EDTA	Ethylenediaminetetraacetic acid
EI	Electron impact
ESI	Electrospray ionization
eV	Electron volt
Fig	Figure
FNR	Ferredoxin-NADP ⁺ reductase
g/L	Grams per liter
GC	Gas chromatography
GC-LRMS	Gas chromatographic-low resolution electron impact mass spectrometric analysis
GC-MS	Gas chromatography-mass spectrometry
gDNA	Genomic deoxyribonucleic acid
h	Hour
HEPES	4-[2-Hydroxyethyl]-1-piperazineethanesulfonic acid
HE-TPP	Hydroxyethyl-thiamine pyrophosphate
HPLC	High-performance liquid chromatography
HS-SPME-GC-TOF-MS	Headspace-solid-phase microextraction-gas chromatography-time-of-flight-mass spectrometry
i.d.	Internal diameter
IMIs	Imidazolinones
IPB	Institute for Plant Biotechnology
IS	Internal standards
ISR	Induced systemic resistance
kDa	Kilodalton
kPa	Kilopascal
kV	Kilovolt
L/h	Liters per hour
LB	Luria-Bertani (medium)
LC-MS	Liquid chromatography-mass spectrometry
m	Meter
M	Molar
m/v	Mass per volume

<i>m/z</i>	Mass-to-charge ratio
mg	Milligram
mg/L	Milligram per liter
min	Minute
mL	Milliliter
mL/min	Milliliters per minute
mm	Millimeter
mM	Millimolar
MS	Murashige and Skoog (medium)
NAD	Nicotinamide adenine dinucleotide
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	Reduced nicotinamide adenine dinucleotide (phosphate)
NADH	Reduced nicotinamide adenine dinucleotide
ng	Nanogram
nm	Nanometer
No.	Number
NPA	1-Naphthylphthalamic acid
OD	Optical density
PCR	Polymerase chain reaction
PDC	Pyruvate decarboxylase
PDMS	Polydimethylsiloxane
pg	Picogram
PGPR	Plant growth promoting rhizobacteria
PGPS	Plant growth promoting substances
PMSF	Phenylmethanesulfonyl fluoride
POBs	Pyrimidinyl oxybenzoates
PVP	Polyvinylpyrrolidone
RID	Refractive index detector
RNA	Ribonucleic acid
rpm	Revolutions per minute
s	Second

SCTs	Sulfonylamino carbonyl triazolinones
SDS	Sodium dodecyl sulfate
SE	Standard error
SEP	Sample enrichment probe
SGD	<i>Saccharomyces</i> Genome Database
SOB	Super optimal broth
sqRT-PCR	Semi-quantitative RT-PCR
SUs	Sulfonylureas
T _m	Melting temperature
TPP	Thiamine pyrophosphate
TPs	Triazolopyrimidines
U/μL	Units per microliter
UPLC	Ultra-performance liquid chromatography
UV	Ultraviolet
V	Volt
v/v	Volume per volume
VOC	Volatile organic compounds
x _g	Relative gravitational force

1. General Introduction and Literature Review

Survival of the fittest is a term used for all living organisms. When considering plants, which are sessile, the fittest are those with the best adaptive plasticity to adapt to changes in the immediate environment. In order to adapt to the changing surroundings, they accumulate storage reserves which will give them the potential to react to these external changes, but by doing this they regulate their growth, even under ideal conditions, to never reach their theoretical maximum potential. In modern farming this growth control is unnecessary and counters maximum production. It is becoming necessary to find novel ways to boost agricultural productivity as extreme pressure is being exerted by the needs of the ever-increasing global population for basic feedstuffs, bio-factories and/or substances for bio-fuels. Recent discoveries led to the finding of several low molecular weight compounds with the ability to stimulate plant growth. None of these compounds fit the profiles of the classical hormone categories. It is still largely unknown how these compounds stimulate plant growth at the physiological level. With few exceptions, the molecular physiology of the growth response has not been examined at all.

Many agricultural practices have been applied and proposed in the past to enhance plant growth via external application of plant growth promoting substances (PGPS) or fertilizers. Certain exogenous substances applied to plants have an effect at a molecular/gene level. In this modern era, as a result of technological advances, it is possible to engineer and introduce foreign metabolic pathways/genes into the desired plants based on the effects exerted by the exogenous applied substances.

In nature, plants are exposed to a number of chemical influences, including those from soil bacteria, also known as rhizobacteria, and various other symbiotic organisms. Some of these chemicals have the potential to act as PGPS and thus modify the growth pattern of the plant. One example of indirectly applying PGPS is the administering of malic acid to the plant or the soil to enhance the occurrence of plant growth promoting rhizobacteria (PGPR) in the rhizosphere (Bais et al., 2014). For the PGPR to be beneficial to the host plant, efficient colonization of the rhizosphere and the rhizoplane is crucial (Compant et al., 2010). A symbiotic mutualism exists between the bacteria and the plant's roots, where the bacteria receive plant root exudates and the plant receives other compounds beneficial for survival, growth or reproduction (Jones et al., 2003).

Rhizobacteria can have a broad range of effects on plants depending on the genus and species. Functions include, but are not limited to: reducing pathogen infection, stimulating other symbioses, increasing nutrient availability in the rhizosphere, nitrogen fixation, phosphate solubilisation, reducing biotic/abiotic plant stress, assisting with iron absorption, increasing yield, positively affecting root growth and stimulating plant growth (Vessey, 2003; Compant et al., 2010).

Rhizobacteria can be classified as PGPR if they affect plants in one or more of the above-mentioned functions (Kloepper and Schroth, 1978), and some of these isolates belong to the genera *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Rhizobium* and *Serratia* (Saharan and Nehra, 2011).

With the above-mentioned positive effects which can be elicited on plants, many agricultural species (i.e. barley, bean, canola, cotton, maize, peanut, rice, vegetables, wheat and woody species) have been treated with rhizobacteria in the past, resulting in increased plant growth (Kloepper et al., 1980; Kloepper et al., 1991).

1.1 Rhizobacteria eliciting plant growth promotion

Various bacterial strains that cover the root surface can secrete metabolites into the surrounding rhizosphere upon consumption of root-released nutrients. These metabolites can be perceived by bacteria or root cells, in close proximity to the secretion site, as signaling compounds (Loon, 2007). The *Rhizobium*-legume symbiosis is a good example of signal exchange. The bacterium is signaled by plant-released flavonoids to secrete Nod factors. Root nodules develop, via the hormone-like effects of the Nod factors, in which atmospheric nitrogen is fixed by the *Rhizobium* bacteria which, in return, receive carbohydrates as a growth substance. The nitrogen can then be used by the plant (Gray and Smith, 2005). Various other bacterial species, other than the *Rhizobium* spp., also have the ability to fix nitrogen (Dobbelaere et al., 2003).

Bacterial siderophores can solubilize poorly soluble inorganic rate-limiting nutrients needed for growth (Vessey, 2003). Phosphate-liberating bacteria produce acids which make phosphate available in poor soil which increases the phosphate availability to plants (Mehta and Nautiyal, 2001). Phosphorus can be made more accessible to plants through a diverse range of bacteria that produce phytases (Idriss et al., 2002) which degrade phytate, to release myo-inositol and free phosphates. Minerals can also be reduced by phenazine-producing rhizobacteria, possibly increasing the range of nutrients available to plants (Hernandez et al., 2004).

Various bacterial species exert growth promoting properties by influencing plant development through the production of growth promoting plant hormones i.e. auxin, cytokinins, ethylene and gibberellins (Loon, 2007). Plant growth promotion by certain bacterial species was due to auxin production, as mutants with low levels of auxin did not stimulate plant growth promotion (Barbieri and Galli, 1993). Ethylene has a couple of possible effects on growth based on its level, ranging from growth promotion at low levels (Pierik et al., 2006) to increased senescence at high levels (Grbic and Bleeker, 1995). Plant roots exude a range of molecules, with amino acids and 1-aminocyclopropane-1-carboxylic acid (ACC), the direct precursor of ethylene, being part of the mixture. Root-produced ethylene can be decreased when re-uptake of ACC is prevented by

rhizobacteria possessing ACC deaminase-activity, resulting in relieving root growth inhibition due to lowered endogenous ACC levels (Glick, 2005).

Lumichrome, a metabolite from riboflavin, can be the product of photo-degraded riboflavin or be synthesized by bacteria (Treadwell and Metzler, 1972). Its effects, being concentration dependent, include enhanced root respiratory rates, increased nutrient and water uptake by the roots, and increased plant size and biomass accumulation (Phillips et al., 1999). The presence of lumichrome in xylem and leaf tissue could possibly be responsible for increased cell division and leaf expansion respectively (Dakora et al., 2001). Lumichrome also affects plants stomatal function which leads to altered leaf transpiration (Matiru and Dakora, 2004). In both *Lotus japonicus* and tomato, lumichrome treatment resulted in the expression of the same 6 genes related to stress and defense (Gouws, 2009).

Certain rhizobacteria are able to produce volatile organic compounds (VOCs) under aerobic and anaerobic conditions. Volatile organic compounds have a high vapor pressure, allowing molecules to evaporate quickly into the air and thereby reach their biological target (Herrmann, 2010). Volatile organic compounds are important chemicals that structure life on earth as they almost have an infinite structural variety based on their function and are found everywhere (Herrmann, 2010). They are produced by humans, animals, plants, fungi, yeast and bacteria (Schulz and Dickschat, 2006) and can cause diverse reactions and act over a wide range of conditions by directly or indirectly influencing plants, insects and humans, and are perfectly adapted to their specific role they have in nature. Some functions include being infochemicals, pheromones, flavors and fragrances (Herrmann, 2010). Yeast and bacteria have been used for many years to produce fermented food with sophisticated aromas which are due to VOCs (Schulz and Dickschat, 2006).

Bacterial VOCs have similar functions as other volatiles in that they can serve as cell-to-cell communication signals, be part of a carbon release valve or have growth inhibiting or promoting properties (Kai et al., 2009). Certain species of rhizobacteria are beneficial to agriculture by directly or indirectly affecting plant growth in a positive manner (Ahmad et al., 2008), but not all rhizobacteria are equally effective in plant growth promotion (Chanway and Nelson, 1991).

Bacterial volatiles are diverse, complex and serve as a deep pool of uncharacterized natural compounds of which the biological functions are not yet understood in detail and thus need to be elucidated further (Kai et al., 2009). Volatile organic compounds can diffuse through aqueous solutions and infuse the air. Thus, VOCs can act both above and below the ground (Kai et al., 2009).

1.2 Plant growth promotion without direct contact with rhizobacteria

Plant growth promoting rhizobacteria can trigger plant growth promotion by releasing volatiles devoid of plant hormones. In 2003, Ryu and colleagues were the first to test how *Arabidopsis thaliana* would react to volatile compounds released from bacteria. Studies conducted since have had contrasting effects, from growth promotion to ultimately plant death. To date, very few molecules released from bacteria have been identified as being exclusively responsible for the observed effects on plant growth. Over 300 possible molecules have been identified in the complex mixtures of volatile blends being released by bacteria, however, a large number of unknown compounds with unknown function were also detected by gas chromatography-mass spectrometry (GC-MS) analyses, which allows for the possibility of discovering new secondary/signaling metabolites (Bailly and Weisskopf, 2012).

Combining the results from numerous studies, it was found that growth promotion was a less frequent observation than growth inhibition caused by bacterial volatile emissions. Closer investigation led to the discovery that the bacterial culture media played an important role in the effects on plant growth. When bacteria were grown on Luria-Bertani (LB) or similar nutrient agar media the volatiles released by the bacteria had a negative effect on plant growth, whereas beneficial effects by the bacteria were only observed when bacteria were grown on Murashige and Skoog (MS) media (Murashige and Skoog, 1962). Based on the different composition between MS and LB (Table 1.1) it is not unexpected that the secondary metabolites produced will differ between the same strain of bacteria and the effects then exerted on plant growth will differ (Bailly and Weisskopf, 2012). When 42 strains of the *Burkholderia* genus were grown on four different media types, it was found that the same strain can have quite different effects, based on the growth medium composition on *A. thaliana* growth (Blom et al., 2011).

Table 1.1: Chemical and physical differences between Murashige and Skoog (MS, 1962) medium and Luria-Bertani (LB) medium.

	MS	LB
Medium composition	Mineral	Complex
Carbon source	Sucrose	Hydrolyzed proteins
pH	Acidic	Slightly alkaline
Agar concentration	Low	High

To establish whether the PGPR released volatile chemicals that effect plant growth via airborne signals and not diffusion through the soil/media, I-plates (petri dishes containing a center partition) were used where *A. thaliana* seedlings were plated on one side of the plate and bacterial cultures on the other side of the I-plate (Ryu et al., 2003). Three out of 7 PGPR tested, *Bacillus amyloliquefaciens* IN937a, *Bacillus subtilis* GB03 and *Enterobacter cloacae* JM22, promoted growth in *A. thaliana* by means of VOCs. These findings suggested that growth promotion by a blend of volatile compounds is not shared by all PGPR for stimulating plant growth (Ryu et al., 2003; Farag et al., 2006). Upon GC-MS analysis of volatiles released from *B. amyloliquefaciens* IN937a and *B. subtilis* GB03, it was shown that both strains emitted high levels of 3-hydroxy-2-butanone, henceforth referred to as acetoin, and 2,3-butanediol. The other gas chromatography (GC) peaks were not consistent between the growth promoting bacteria. Plant growth promoting rhizobacteria lacking plant growth promotion via VOCs were deficient in acetoin and 2,3-butanediol production, suggesting that bioactive VOC synthesis is strain-specific (Ryu et al., 2003; Farag et al., 2006). When captured volatiles from *B. amyloliquefaciens* IN937a and *B. subtilis* GB03 were tested against *A. thaliana* they significantly increased the total leaf surface area compared to the controls (Ryu et al., 2003). Mutant strains of *B. subtilis* devoid of the 2,3-butanediol producing pathway were compared against wild type *B. subtilis* to confirm the necessity/efficiency of 2,3-butanediol for plant growth promotion; the mutant lines did not produce a growth promoting effect. To back up these findings, commercially available synthetic 2,3-butanediol were also tested against *A. thaliana* for plant growth promotion and a dose-responsive growth curve was observed (Ryu et al., 2003).

When *Burkholderia* strains were grown on Methyl-Red-Voges-Proskauer media, most of them promoted growth in *A. thaliana*. The media favors the production of 2,3-butanediol fermentation, however, the *Burkholderia* genus does not contain the pathway for 2,3-butanediol production, which suggests that other volatiles are responsible for the growth promotion in these strains. To date only two other compounds i.e. dimethylhexadecylamine (Velazquez-Becerra et al., 2011) and 2-pentylfuran (Zou et al., 2010) have caused plant growth promotion when applied as pure substances. In other studies hydrogen cyanide (Blom et al., 2011), dimethyl disulfide and NH₃ (Kai et al., 2010) have been identified as substances that have a deleterious effect on plants. The above-mentioned compounds have a definitive effect on plant growth individually, but it is more likely that a mixture of compounds (including unknown/novel compounds) work together for growth promotion or growth inhibition (Bailly and Weisskopf, 2012).

In addition to growth promoting volatile production by bacteria, the time point after germination at which the plants are exposed to volatiles also greatly influences the outcome of the interaction. Fresh weight and root length of *Medicago sativa* increased significantly compared to controls when the seedlings were exposed to VOCs from *Arthrobacter agilis* UMCV2 at 24 and 48 h after

germination, however, no significant changes were observed at 0, 72 and 96 h after germination (Velazquez-Becerra et al., 2011).

To gain a better understanding of the mechanisms involved in plant growth promotion due to PGPR that emit bioactive VOCs, *B. amyloliquefaciens* IN937a and *B. subtilis* GB03 were tested against *A. thaliana* mutants that were defective in specific phytohormones regulatory pathways. Results showed that growth promotion activated by VOC exposure is not by means of the brassinosteroid-, ethylene- or gibberellic acid-signaling pathways but could be due to the cytokinin-signaling pathways (Ryu et al., 2003).

1.3 Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion

Further studies were conducted by Zhang et al. in 2007 where oligonucleotide microarrays were used to examine the ribonucleic acid (RNA) transcript levels of *Arabidopsis* plantlets exposed to *B. subtilis* GB03 VOCs, by screening the complete genome consisting of 26 751 genes. An average of 648 genes were differentially expressed after *B. subtilis* GB03 exposure, with 56% of the genes being up-regulated and 44% being down-regulated. These genes were mainly related to cell wall modifications, primary and secondary metabolism, stress responses, hormone regulation and elevated protein synthesis (Zhang et al., 2007).

Transcriptional and histochemical data indicated that growth promotion was due to auxin homeostasis as a result of the differentially expressed auxin synthesis genes being up-regulated at least once, either at 48 or 72 h measurement after exposure to *B. subtilis* GB03 VOCs. Activation of basipetal auxin transport was suggested as a transgenic DR5::GUS *Arabidopsis* line exposed to *B. subtilis* GB03 VOCs revealed that auxin synthesis was up-regulated in leaves, however, auxin accumulated in roots and decreased in leaves. Auxin transport was inhibited by applying 1-naphthylphthalamic acid (NPA) which resulted in auxin accumulation in the leaves and prevented *B. subtilis* GB03-mediated growth promotion. Lateral root formation and leaf expansion thus appears to result from the regulation of auxin levels in the specific plant tissues by the growth promoting VOCs from the *B. subtilis* GB03 strain (Zhang et al., 2007).

With *B. subtilis* GB03 exposure, microarray data revealed that approximately 5% of all differentially regulated genes were related to cell wall modification and that these were mostly up-regulated. Roughly 80% of the differentially regulated genes with known functions to cell-wall structure were coordinately regulated to expand cells. Cytological measurements confirmed cell expansion in leaf samples but no significant differences were observed in the primary or lateral root cells, however, the number of lateral roots of *B. subtilis* GB03 treated plants increased significantly compared to the water controls (Dubrovsky et al., 2001; Zhang et al., 2007).

1.4 Sustained growth promotion

It was found that constant exposure of *B. subtilis* GB03 volatile emissions were necessary for continuous growth promotion in *A. thaliana* compared to water controls, as early withdrawal of *B. subtilis* GB03 exposure in plant development resulted in the loss of enhanced growth (Xie et al., 2009). Plant growth promotion resulted from a greater number of rosette leaves as well as higher fresh and dry masses. Delayed flowering was observed, however, silique/seed number was significantly higher than water controls upon harvest (Xie et al., 2009). Chlorophyll content, photosynthetic rates and iron levels also increased under continuous exposure (Zhang et al., 2008; Xie et al., 2009), but when volatile exposure was withdrawn the increased photosynthetic capability and iron levels returned to untreated levels (Xie et al., 2009). Within a period of three days following *B. subtilis* GB03 volatile exposure, differential transcriptional expression of genes involved with iron regulation and cell wall functions were triggered but the effect was brief as these levels returned to those of water-treated controls within one week after *B. subtilis* GB03 exposure. Thus, post-transcriptional mechanisms, at least for the above-mentioned genes, are suggested to play a role in sustaining *B. subtilis* GB03 volatile growth promotion (Xie et al., 2009).

1.5 Induced systemic resistance

Non-pathogenic rhizobacteria can indirectly promote plant growth by altering the plant to increase its defense systems or by antagonizing pathogens (Van Loon and Bakker, 2003). The former method is better known as induced systemic resistance (ISR). Just over 20 years ago it was found that carnation and cucumber were systemically protected against pathogen infection upon treatment with non-pathogenic rhizobacteria including *Pseudomonas* spp. (Van Peer et al., 1991; Wei et al., 1991). The protective effect conferred by the non-pathogenic rhizobacteria was plant-mediated as the pathogen infection remained confined to the point of infection and had no physical interaction with the non-pathogenic rhizobacteria. Induced systemic resistance increases the plant's defensive capacity by reducing the rate of disease development (Van Loon et al. 1998; Van Loon and Bakker, 2005). Seedlings are at a vulnerable stage during development and are a target for infectious microorganisms such as *Phythium*, *Fusarium* or *Rhizoctonia* which causes damping-off of the seedlings resulting in death or reduced growth. Rhizobacteria can protect the plants by promoting growth which will shorten the vulnerable stage and result in reduced disease severity. Another inducible defense mechanism, known as priming, occurs when non-pathogenic rhizobacteria activate the plants' defenses in a similar way to pathogenic infection or enhance the capacity of the plants' defenses upon pathogenic infection (Conrath et al., 2006).

The disease severity on *A. thaliana* caused by *Erwinia carotovora* subsp. *carotovora* was significantly reduced by activation of ISR due to VOC exposure from *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a in as little as 4 days (Ryu et al., 2004). Induced systemic resistance of

A. thaliana against *Pseudomonas syringae* pv. tomato DC300 was also caused by *B. subtilis* FB17 (Rudrappa et al., 2010). Exogenous application of acetoin or 2,3-butanediol confirmed the involvement of these two volatiles in triggering ISR. Transgenic and mutant *B. subtilis* lines, with a reduced ability to synthesize acetoin or 2,3-butanediol, were compared against wild type *B. subtilis* in their ability to induce systemic resistance upon pathogen infection. Results indicated that the transgenic and mutant lines had reduced ISR effects on *A. thaliana* compared to that initiated by wild type *B. subtilis* (Ryu et al., 2004; Rudrappa et al., 2010). Thus, as discussed above, the volatiles released by *B. subtilis* (i.e. acetoin and 2,3-butanediol) are responsible for not only triggering plant growth promotion (Ruy et al., 2003) but eliciting ISR in *A. thaliana* as well (Ryu et al., 2004; Rudrappa et al., 2010).

1.6 Synthesis of acetoin and 2,3-butanediol

1.6.1 Bacteria

Acetoin, a four-carbon volatile alcohol (Ryu et al., 2003), is produced by a wide range of microorganisms including, but not limited to, *B. subtilis*, *Lactococcus lactis* and *Lecucostoc mesenteroides* (Bassit et al., 1995; Schmitt et al., 1997; Huang et al., 1999) in their respective fermentative metabolisms. It is a very crucial physiological metabolite which is an intermediate product in the production of 2,3-butanediol (Xu et al., 2011). Acetoin and 2,3-butanediol share a very similar structure (Fig 1.1). Acetoin production has mainly three roles: avoiding acidification, regulating the nicotinamide adenine dinucleotide (NAD) / reduced nicotinamide adenine dinucleotide (NADH) ratio and serving as an energy source or storage carbon for growth (Huang et al., 1999).

Under anaerobic fermentation, cytoplasmic acidification may occur. To neutralise the pH, a catabolic acetolactate synthase (ALS, EC 2.2.1.6) is expressed which converts two molecules of pyruvate to one molecule of α -acetolactate. The α -acetolactate is then converted by α -acetolactate decarboxylase (ALDC, EC 4.1.1.5) to a neutral product, acetoin, that can either be excreted or reversibly transformed (Xu et al., 2011) by acetoin reductase/2,3-butanediol dehydrogenase (BDH1, EC 1.1.1.4) to 2,3-butanediol, which in turns regulates the NAD/NADH ratio (Forlani, 1998; Xu et al., 2011). The α -acetolactate can also turn into diacetyl by spontaneous decarboxylation. Diacetyl (Fig 1.1) can in turn be converted to acetoin by diacetyl reductase (EC 1.1.1.303) or 2,3-butanediol dehydrogenase (Fig 1.2; Xu et al., 2011).

The α -acetolactate molecule can have three functions. Firstly it can be converted to acetoin or secondly to diacetyl as mentioned above. Thirdly it is also a direct intermediate in the synthesis of two of the three branched-chained amino acids (BCAA), leucine and valine. It was shown that there are two α -acetolactate-forming enzymes present in bacteria i.e. the anabolic α -acetolactate

synthase and the catabolic α -acetolactate synthase (Halpern and Even-ahoshan, 1967; Mallonee and Speckman, 1988) with the anabolic α -acetolactate synthase being involved in the synthesis of the BCAA (Xu et al., 2011) and the latter in the formation of acetoin.

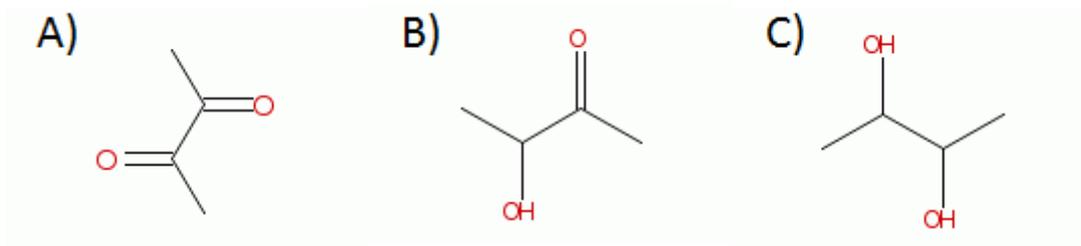


Figure 1.1: A) diacetyl; B) acetoin; C) 2,3-butanediol (Chemical structures prepared from <http://web.chemdoodle.com/demos/sketcher>; <https://www.emolecules.com>).

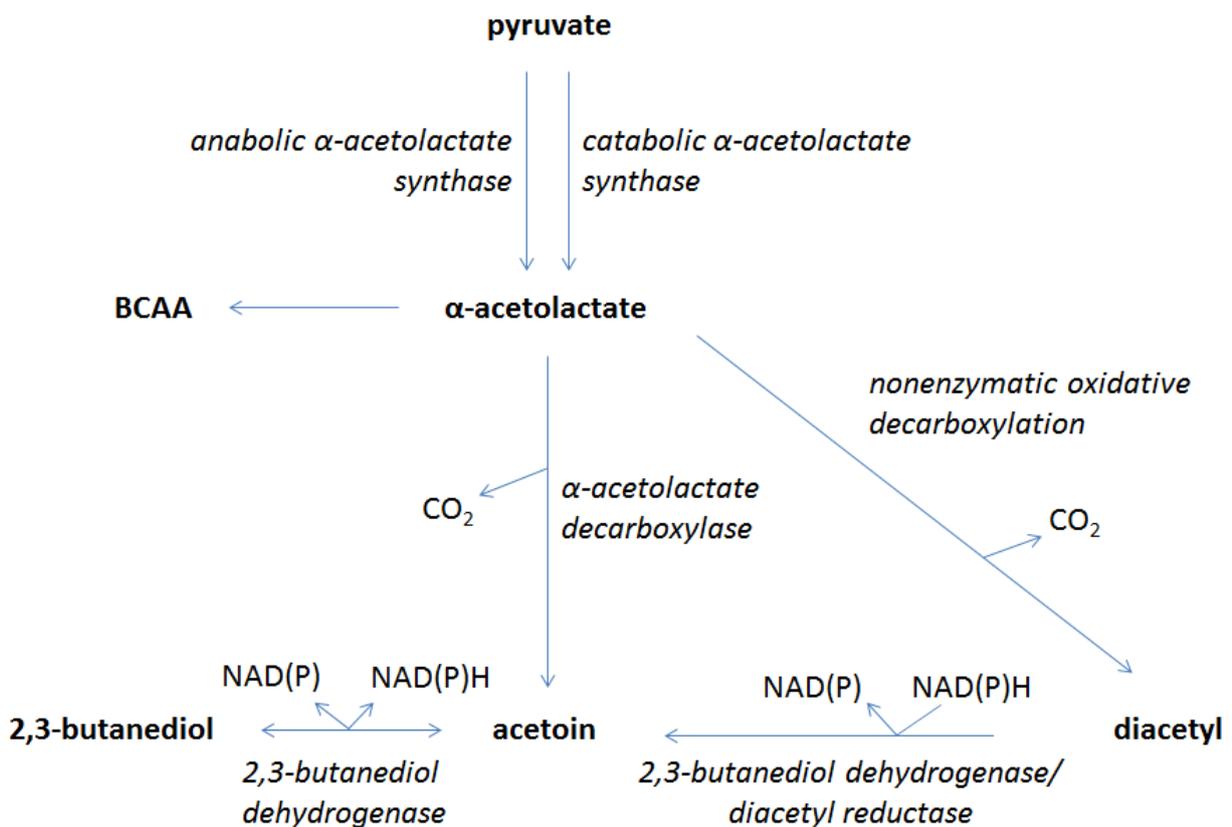


Figure 1.2: Biosynthetic pathway of acetoin and 2,3-butanediol in bacteria, BCAA, branched chain amino acids; NAD(P), nicotinamide adenine dinucleotide (phosphate); NAD(P)H, reduced nicotinamide adenine dinucleotide (phosphate); CO_2 , carbon dioxide (modified from Xu et al., 2011).

1.6.2 Yeast

In yeast, acetoin can be produced from either acetaldehyde or pyruvate by pyruvate decarboxylase (PDC, EC 4.1.1.1) or from diacetyl by diacetyl reductase (EC 1.1.1.303). As in bacteria, acetoin can be further converted to 2,3-butanediol by 2,3-butanediol dehydrogenase (EC 1.1.1.4).

1.6.3 Plants

It has been shown that certain plants can also produce acetoin, but by a different mechanism than in bacteria. As mentioned above, bacteria can either form acetoin from α -acetolactate by the enzyme α -acetolactate decarboxylase (ALDC, EC 4.1.1.5) or by first forming diacetyl by a non-enzymatic reaction which is then converted to acetoin by diacetyl reductase (EC 1.1.1.303) or 2,3-butanediol dehydrogenase (EC 1.1.1.4).

Two different enzymatic activities were detected in plant cell cultures of carrot, maize, rice and tobacco which were able to generate acetoin, namely a side reaction of PDC and pyruvate carboligase (putative, no EC number) (Forlani et al., 1999). In addition to proliferating cell cultures, acetoin synthesis by PDC has also been detected in plant tissue from wheat germ (Singer and Pensky, 1952) and ripening pea seeds (Davies, 1964). Acetoin is formed by PDC via direct condensation of an acetaldehyde moiety with hydroxyethyl-thiamine pyrophosphate (HE-TPP) that is bound as an intermediate to the enzyme (Juni, 1961; Chen and Jordan, 1984). Acetoin synthesis was only detected when pyruvate or acetaldehyde were present as substrates, while no acetoin production was detected in crude extracts in which acetolactate was supplied as the substrate. One mole of acetoin was produced for every two moles of pyruvate when acetaldehyde was absent, whilst in the presence of acetaldehyde, one mole of acetoin was produced per mole of pyruvate (Forlani et al., 1999). In *Zea mays*, after an initial lag, the two enzymes (PDC and pyruvate carboligase) had activity with acetaldehyde alone as well as higher rates with pyruvate alone. Pyruvate can be condensed into acetoin even when it is lacking two acetaldehyde groups due to the reversibility of the reaction ($\text{HE-TPP} \leftrightarrow \text{acetaldehyde} + \text{TPP}$) (Juni, 1961; Chen and Jordan, 1984). When thiamine pyrophosphate (TPP) and divalent cations (Mg^{2+} or Mn^{2+}) were omitted from the substrate, no pyruvate carboligase activity was detected (Forlani et al., 1999). Acetaldehyde synthesis by PDC occurs at much higher rates than acetoin synthesis. In the presence of pyruvate alone, low levels of acetaldehyde were released in the initial stages of incubation. The acetaldehyde levels remained stationary as additional acetaldehyde was most likely condensed to acetoin as soon as it was produced by the putative carboligase (Forlani, 1999). Acetoin is not only the end product of α -acetolactate decarboxylase but also a synthesized side-product of most TPP-dependant decarboxylating enzymes (Chen and Jordan, 1984; Bertagnolli and Hager, 1993). The 2,3-butanediol pathway is exclusive to certain bacteria, yeast and fungi and does not exist in plants. Thus, the significant levels of acetoin produced from the above-mentioned plant crude extracts are only possible due to a side reaction of PDC (Shimizu et al., 1994). Acetoin

production may be the end result of a detoxifying pathway for acetaldehyde as plants cells are quickly negatively influenced by the presence thereof. The putative carboligase has a high affinity for acetaldehyde and, as a result, a rapid non-toxic conversion of acetaldehyde takes place with acetoin being the end product (Forlani, 1999).

To date no studies have been conducted on *A. thaliana* for the detection of acetoin, but could be of value as *A. thaliana* is a model plant for studies. Previous results indicated that exogenous application of acetoin/2,3-butanediol have growth promoting and ISR properties on *A. thaliana* (Ryu et al., 2004; Rudrappa et al., 2010), thus incorporating the production of acetoin/2,3-butanediol *in planta* could possibly have similar effects.

1.7 Aim of the project

This study was undertaken with the intention to enhance plant growth by means of incorporating genes responsible for the production of two volatile organic compounds i.e. acetoin and 2,3-butanediol into the model plant *A. thaliana*. *Arabidopsis thaliana* plants exposed to the volatile emissions of *Bacillus subtilis* GB03 have been shown to display long-term growth promotion, in the form of enhanced vegetative growth followed by elevated seed set, in comparison with control plants (Ryu et al., 2003; Ryu et al., 2004). The continued presence of the volatiles was shown to be important, as early withdrawal of the bacteria (releasing the volatiles) during plant development resulted in plants that were not significantly larger than the control plants (Xie et al., 2009).

The approach of this study was to construct two separate vectors with constitutive promoters to transfer the fungal gene α -acetolactate decarboxylase (*ALDC*) and the yeast gene 2,3-butanediol dehydrogenase (*BDH1*), responsible for the production of acetoin and 2,3-butanediol respectively, into *A. thaliana* by means of the *Agrobacterium*-mediated floral dip method, in order to overexpress these organic volatile compounds *in planta*. The transformants were then screened for gene incorporation and expression as well as the production of functional proteins with the hope of enhancing general plant growth and seed production.

2. Materials and Methods

2.1 Chemicals

All chemicals, unless specified otherwise, were of molecular biology grade and obtained from Sigma Aldrich Fluka (St. Louis, MO, USA), Merck (Wadeville, Gauteng, RSA) and Promega (Madison, WI, USA). All nucleic acid modifying enzymes, unless specified otherwise, were from Fermentas and were obtained from Inqaba Biotec™ (Inqaba Biotechnical Industries, RSA). All primers used during this study were synthesised by Inqaba Biotechnical Industries.

2.2 Plant tissue culture and growth conditions

Arabidopsis thaliana Columbia-0 seeds were surface decontaminated for 5 min in 70% (v/v) ethanol, followed by 5 min in 1.75% (m/v) sodium hypochlorite (Chlor Guard®) containing two drops Tween-20 per 20 mL. The seeds were rinsed 5 times in sterile de-ionised distilled water (ddH₂O) before being stratified for 3 days in the dark at 4°C whilst submerged in ddH₂O.

Surface decontaminated *A. thaliana* seeds were plated on half-strength MS medium (Highveld Manufacturing) with the addition of 3% (m/v) sucrose and solidified with 7 g/L bacteriological agar in Cellstar® 100 x 20 mm cell culture dishes (Greiner Bio-One). Media was prepared by adjusting the pH to 5.8 using KOH before adding agar and autoclaving at 121°C, 100 kPa for 20 min.

Cell culture plates were incubated horizontally or vertically, depending on the experiment, and the seedlings were germinated and grown at 25±2°C for 14 d, post-germination, in a 16h:8h light:dark photoperiod, under cool, white fluorescent tubes (Osram L 58V/740) with a light intensity of 50 µmoles photons.m⁻².s⁻¹.

2.3 Yeast and fungal growth conditions

Axenic fungal (*Aspergillus niger* ATCC 10864) and yeast (*Saccharomyces cerevisiae* W303) cultures were streaked out onto an 80 mm 325P cellophane disc (A.A. Packaging, UK) laid out on solid YPD media (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrin, 15 g/L bacto-agar) in 90 x 15 mm petri dishes. Fungal cultures were incubated for 14 d at 28°C in the dark, while yeast cultures were incubated for 2 d under the same conditions before being used for extractions, after which they were stored at 4°C for up to 1 month prior to sub-culturing.

2.4 Isolating the α -acetolactate decarboxylase (*ALDC*) cDNA from *Aspergillus niger*

Since the sequence data for the α -acetolactate decarboxylase (*ALDC*, EC 4.1.1.5) gene (An03g00490) in the *Aspergillus* Genome Database (AspGD, <http://www.aspergillusgenome.org>) indicated the presence of an intron, total RNA was extracted from *Aspergillus niger* ATCC 10864 using the RNeasy[®] Plant Mini Kit (QIAGEN) as per manufacturer's specifications. The RNA was quantified by a NanoDrop ND 1000 (Thermo Fisher Scientific) spectrophotometer and evaluated on a 2% agarose gel stained with ethidium bromide. Complementary deoxyribonucleic acid (cDNA) was synthesised from 1 μ g total RNA with the RevertAid[™] H First strand Synthesis cDNA kit (Fermentas) using Oligo-dT₁₈ primers as specified by the manufacturer. Isolated cDNA was stored at -80°C.

2.5 Isolating the acetoin reductase/2,3-butanediol dehydrogenase (*BDH1*) gene from *Saccharomyces cerevisiae*

Genomic deoxyribonucleic acid (gDNA) was isolated from *Saccharomyces cerevisiae* W303 using a method modified from Rose et al. (1990) as the acetoin reductase/2,3-butanediol dehydrogenase (*BDH1*, EC 1.1.1.4) gene contained no intron regions. The yeast cell culture was transferred to a 2 mL micro-centrifuge tube and centrifuged for 5 min at 16 000 xg to pellet the cells. After removal of the supernatant, 200 μ L glass beads (425 – 600 μ m), 200 μ L lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM ethylenediaminetetraacetic acid [EDTA] pH 8.0, 100 mM NaCl, 1% [m/v] sodium dodecyl sulfate [SDS], 2% [v/v] Triton X-100) and 200 μ L phenol:chloroform (1:1) were added to the tubes. The tubes were vortexed at top speed for 1 min and placed on ice for 1 min. This cycle was repeated another four times. Two-hundred μ L of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0) was added to each tube. This was vortexed for a few seconds and then centrifuged for 5 min at 16 000 xg at room temperature. The supernatant (300 μ L) was transferred to a new 1.5 mL micro-centrifuge tube and two volumes of absolute ethanol at room temperature were added and the samples gently mixed. The tubes were centrifuged for 3 min at room temperature and the supernatant was then aspirated. The resulting pellet was washed with 70% (v/v) ice-cold ethanol and centrifuged at 16 000 xg for 1 min at room temperature. The supernatant was discarded before the pellets were dried in a laminar flow-hood for 30 to 60 min and resuspended in 30 μ L ddH₂O. Isolated gDNA was quantified by a NanoDrop ND 1000 (Thermo Fisher Scientific) spectrophotometer before being stored at -20°C.

2.6 Amplification using polymerase chain reaction

The gene sequences of *α-acetolactate decarboxylase (ALDC)* from *AspGD* and *acetoin reductase/2,3-butanediol dehydrogenase (BDH1)* from Saccharomyces Genome Database (SGD) were used to design primers (Table 2.1) for polymerase chain reaction (PCR) amplification. Primers were designed with specific restriction sites (Table 2.1) flanking the genes for easier insertion into the targeted vectors so that the genes would remain in frame with the FNR sequence. Amplification was performed using the Kapa HiFi™ PCR kit (Kapa Biosystems). The reaction was set up as per the manufacturer's recommendation with 100 ng of gDNA or 1 μL cDNA being used during the PCR. The recommended cycling protocol for Kapa HiFi™ was used in a GeneAmp® PCR System 9700 (Applied Biosystems) thermocycler under the conditions described in Table 2.2.

Table 2.1: Primer sequences for the amplification of the *ALDC* and *BDH1* genes with restriction sites.

Primer name	Sequence	RE Sites	Product Size
ALDC_FW	5'-GAGCTCTATGGAGACATGGGTATCACA-3'	<i>SacI</i>	960 bp
ALDC_RW	5'-AAGCTTTTAGTGAGAAGTGGGGACTCC-3'	<i>HindIII</i>	
BDH1_FW	5'-GTCGACTTATGAGAGCTTTGGCATATTTCAAG-3'	<i>SalI</i>	1149 bp
BDH1_RW	5'-GAGCTCTTACTTCATTTACCGTGATTGTTAG-3'	<i>SacI</i>	

Table 2.2: PCR cycling protocol for Kapa HiFi™ of the *ALDC* and *BDH1* genes.

PCR step	Cycling conditions		No. of cycles
	Temperature	Time	
Initial denature	95°C	3 min	1
Denature	98°C	20 s	
Annealing (T _m)	65°C	15 s	25
Extension	72°C	40 s	
Final elongation	72°C	1 min	1

2.7 Separation of DNA fragments by gel electrophoresis

Between 1 and 5 μL of each of the PCR amplification products were electrophoresed on an agarose gel (1% [m/v] agarose, 0.5x TBE buffer [5.4 g/L Tris base, 2.75 g/L Boric acid, 0.465 g/L EDTA pH 8.0]) stained with 2.5 μL/50 mL Pronasafe (Laboratorios Conda). The gel was submerged in 0.5x TBE buffer and deoxyribonucleic acid (DNA) fragments were separated within the gel at 100 V for 40 min. Separated fragments were viewed under ultraviolet (UV)-light using the

Alpha Imager 2000 (Alpha Innotech). The PCR products were purified directly from the amplified product using the GeneJET™ PCR Purification Kit (Thermo Fisher Scientific) as specified by the manufacturer.

2.8 Preparation of chemically competent *Escherichia coli* DH5α cells

Escherichia coli DH5α cells were inoculated in 2 mL liquid LB media (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl). The culture was incubated at 37°C with shaking at 200 rpm overnight. One mL of the overnight culture was inoculated into 125 mL of liquid super optimal broth (SOB) media (5 g/L Yeast Extract Powder, 20 g/L Tryptone, 10 mM NaCl, 2.5 mM KCl, 20 mM MgSO₄ pH 7.5) and grown to an optical density (OD) of 0.3 at 600 nm. The cells were brought to 4°C before being centrifuged at 1500 *xg* for 10 min at 4°C. The supernatant was aspirated and the pelleted cells were gently resuspended in 40 mL of ice cold CCMB80 buffer (10 mM KAc pH 7.0, 80 mM CaCl₂·2H₂O, 20 mM MnCl₂·4H₂O, 10 mM MgCl₂·6H₂O, 10% [v/v] glycerol, pH 6.4) and incubated on ice for 20 min. The resuspended cells were centrifuged at 1500 *xg* for 10 min at 4°C. After removal of the supernatant the pelleted cells were resuspended in 10 mL of CCMB80 buffer. The resuspended cells were diluted with CCMB80 buffer until a mixture of 50 µL of the resuspended cells and 200 µL LB broth yielded an OD_{600 nm} of between 1.0-1.5. Fifty µL resuspended cells were snap frozen in liquid nitrogen and stored at -80°C.

2.9 Ligation of PCR products and selection of plasmids containing inserts

The purified PCR products were ligated into the pJet1.2/blunt cloning vector (Fig 2.1) as per the CloneJet™ PCR Cloning Kit (Thermo Fisher Scientific) specifications for blunt end ligations. The ligation reaction was transformed into chemically competent *E. coli* DH5α by a heat shock method, using the competent cells that were prepared as previously described. During heat shock transformation, 5 µL of the pJet1.2/blunt ligation was added to 50 µL ice-thawed competent cells and placed on ice for 20 min. The cell-ligation mixture was then incubated at 42°C for 60 s and placed back on ice for a further 2 min before adding 945 µL liquid LB media. The transformation culture was incubated at 37°C with shaking at 200 rpm for 2 h. Two-hundred µL of transformed culture was spread out on solid LB media containing 50 µg/mL ampicillin. Plates were incubated at 37°C for 16 h. Putative positive colonies were selected based on the ability to grow in the presence of ampicillin and used as template for colony PCR with the relevant gene-specific primers (Table 2.1) to confirm the insertion of the PCR-gene product into the pJet1.3/blunt vector. One µL of each of the colony PCR amplification products were separated on a 1% (m/v) agarose gel. Colonies which gave rise to amplification products of the expected size were inoculated in 3 mL LB media containing 50 µg/mL ampicillin and grown overnight at 37°C with shaking at 200 rpm.

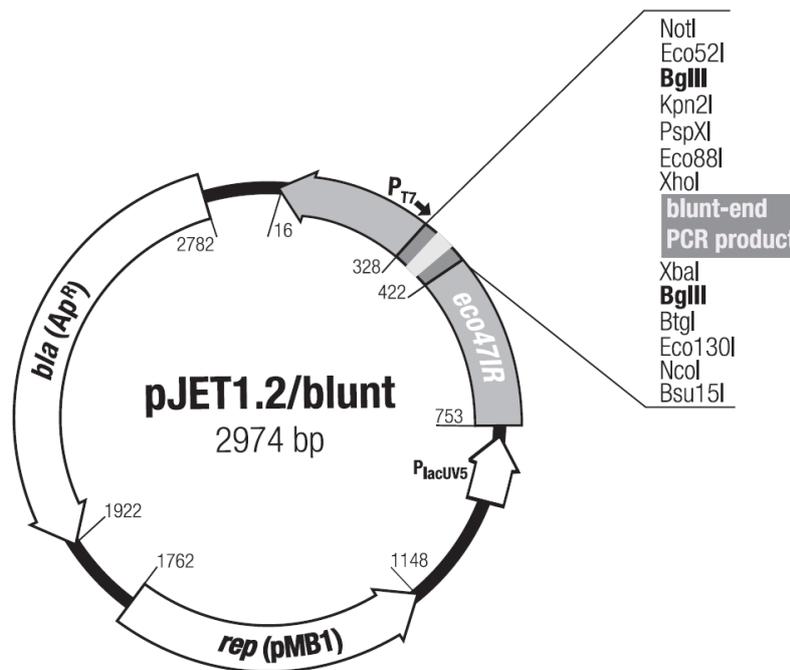


Figure 2.1: pJet1.2/blunt cloning vector map (Thermo Fisher Scientific).

2.10 Isolation of plasmid DNA

Bacterial cultures were centrifuged at 16 000 xg for 2 min. After aspirating off the supernatant, the resulting pellet was re-suspended in 200 μ L Solution I buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.1 g/L RNase A) by vortex. Two-hundred μ L Solution II buffer (200 mM NaOH, 1% [m/v] SDS) was added and the tubes were gently mixed by inversion before 200 μ L Solution III buffer (3 M potassium acetate, pH 5.5) was added and gently, but thoroughly, mixed by inversion. The mixture was cooled on ice for 5 min and centrifuged at 16 000 xg for 10 min, after which the supernatant was removed and added to 450 μ L ice-cold isopropanol in a fresh tube. The suspension stood on ice for 5 min to precipitate the plasmid DNA, which was collected by centrifugation at 16 000 xg for 10 min. The supernatant was discarded and the pellet washed with 1 mL 70% (v/v) ethanol and centrifuged for 5 min at 16 000 xg . After discarding the supernatant, the pellet was air dried in a laminar flow-hood for 30 to 60 min and resuspended in 30 μ L ddH₂O and purified using the GeneJET™ PCR Purification Kit (Thermo Fisher Scientific), as per manufacturer's specifications.

2.11 Sequence confirmation

The plasmid DNA was quantified with a NanoDrop ND 1000 (Thermo Fisher Scientific) and sent for bidirectional sequencing at the Central Analytical Facility (CAF), Stellenbosch University, with the

pJet1.2 forward (5'-CGACTCACTATAGGGAGAGCGGC-3') and reverse (5'-AAGAACATCGATTTTCCATGGCAG-3') primers.

The data received from CAF was aligned with the gene sequences from AspGD and SGD in ClustalW (Larkin et al., 2007) and displayed in JalView (Waterhouse et al., 2009).

2.12 Modifying transformation constructs

Correct sequences were backtracked to the corresponding plasmids. Plasmid DNA was double digested with restriction enzymes *SacI* and *HindIII* for the *ALDC* gene or, *SaI* and *SacI* for the *BDH1* gene according to the manufacturer's instructions (Fermentas) to excise the inserts using the restriction sites built into the primers, to allow for directional cloning into the pCambia vectors. The digested samples were run on a 1% (m/v) agarose gel. Correct DNA bands were cut from the agarose gel using a fresh sterile scalpel blade for each band and purified from the gel using a GeneJET™ Gel Extraction Kit (Thermo Fisher Scientific) according to the manufacturer's specifications.

The purified genes, *ALDC* and *BDH1*, were ligated into pCambia2300::*FNR* and pCambia1300::*FNR* respectively, as per the T4 DNA Ligase Kit (Thermo Fisher Scientific) specifications for a sticky-end ligation, with the exception of a 1 h incubation instead of 10 min for better ligation. The pCambia1300 and pCambia2300 vectors differ from each other based on the plant selection genes which made it easier for selection of putatively double transformants after transformation. The vectors were modified and given as a kind gift, by Dr James Lloyd (Institute for Plant Biotechnology (IPB), Stellenbosch), by taking an *EcoRI/HindIII* fragment containing the 35S promoter, polylinker and terminator from pBINAR (Höfgen and Willmitzer, 1990) containing the ferredoxin-NADP⁺ reductase (FNR) chloroplastic transit peptide sequence from *Spinacia oleracea*, and ligating that into the *EcoRI/HindIII* sites of the relevant pCambia vectors.

The pCambia constructs were heat shocked as previously described with the exception that the transformed culture was spread onto solid LB media containing 50 µg/mL kanamycin instead of ampicillin. Plates were incubated at 37°C for 16 h. Putative positive colonies were selected and used as template for colony PCR with gene-specific primers (Table 2.1) to confirm the presence of the correctly sequenced gene product into the constructs.

One µL of each of the colony PCR amplification products were separated on a 1% (m/v) agarose gel. Colonies which gave rise to amplification products of the expected size were inoculated in 3 mL LB medium containing 50 µg/mL kanamycin and grown overnight at 37°C with shaking at 200 rpm. Bacterial cultures were used for isolation of constructs as previously described and quantified

by a NanoDrop ND 1000 (Thermo Fisher Scientific) spectrophotometer before being stored at -20°C.

2.13 *Agrobacterium tumefaciens* plasmid transformation

The plasmids containing the *ALDC* and *BDH1* genes were transformed into *Agrobacterium tumefaciens* GV3101, using a modified heat shock method from www.ivaan.com/protocols/160.html. Ten mL LB with 25 µg/mL Geneticin G418 and 50 µg/mL rifampicin were inoculated with *A. tumefaciens* GV3101 and incubated overnight in the dark at 28°C with shaking at 240 rpm. Two mL aliquots of the overnight culture were transferred to 2 mL micro-centrifuge tubes and chilled on ice for 10 min. The ice-cold aliquots were then centrifuged at 4°C for 3 min at 16 000 *xg*. After aspiration of the media, the pelleted cells were gently resuspended in 1 mL ice-cold 10 mM Tris-HCl (pH 7.5). The cell mixture was then put back on ice for 10 min and centrifuged at 4°C for 3 min at 16 000 *xg*. After removal of the supernatant the cells were resuspended in 200 µL ice-cold liquid LB and 2 µg of previously prepared pCambia constructs were added, ending up with two separate plasmids, pCambia2300::*FNR:ALDC* and pCambia1300::*FNR:BDH1*. The cell and plasmid mixture was frozen in liquid nitrogen for 5 min and immediately incubated at 37°C for 5 min. To recover the cells, 1 mL of LB was added to the tubes which were incubated at 28°C with shaking at 240 rpm for 3 h. Transformed culture (200 µL) was spread out on solid LB media containing 25 µg/mL Geneticin G418, 50 µg/mL rifampicin and 50 µg/mL kanamycin. Plates were incubated at 28°C for 2 days. Random colonies were picked and inoculated in 3 mL liquid LB media containing 25 µg/mL Geneticin G418, 50 µg/mL rifampicin and 50 µg/mL kanamycin and grown overnight at 28°C with shaking at 240 rpm. Plasmids were isolated from bacterial cultures using 2 mL cultures as previously described and subsequently used for plasmid PCR with gene-specific primers (Table 2.1) to confirm the presence of the gene product. Glycerol stocks were prepared from positive cultures by adding 800 µL of the remaining bacterial culture to 800 µL 80% (v/v) autoclaved glycerol in sterile Cryo Tubes™ (Thermo Fisher Scientific) and frozen in liquid nitrogen before being stored at -80°C.

2.14 Growing *Arabidopsis thaliana* for floral-dipping

Surface decontaminated *A. thaliana* seeds were plated onto tissue culture growth media and grown in tissue culture conditions as previously described. After the growth period, the plantlets were carefully removed from the plates and potted in a soil mixture consisting of a 4:4:1:1 ratio of Vermigo:palm-peat:vermiculite:quartz sand and grown for three to four weeks, to establish a strong vegetative state, in a 10h:14h light:dark photoperiod, with a light intensity of 90 µmoles photons.m².s⁻¹ at a 21°C:15°C day:night temperature range with humidity averaging 90%. The

plants were watered by sub-irrigation every 3 to 4 d. Any inflorescences that emerged during this time period were cut off at the base of the rosette. Once the plants had grown sufficiently, they were moved to a greenhouse with a longer day length (14h:10h light:dark photoperiod) to initiate bolting. Inflorescences were trimmed twice over a two week period to induce more prolific flowering. Once the inflorescences reached 15 cm in height, these were used for *A. tumefaciens*-mediated transformation via the floral dipping method.

2.15 *Agrobacterium*-mediated floral dipping transformation

Arabidopsis thaliana plants were transformed using a modified *Agrobacterium*-mediated floral dip method (Zhang et al., 2006). Previously prepared glycerol stocks were used to inoculate 200 mL liquid LB containing the appropriate antibiotics. Cultures were grown in the dark at 28°C whilst shaking at 240 rpm for 16-24 h until an OD_{600 nm} of between 1.5 and 2.0 was reached. *Agrobacterium* cultures were centrifuged at 4000 *xg* for 10 min at room temperature, after which the supernatant was removed and the cells were gently resuspended in 1 volume of a fresh 5% (m/v) sucrose solution by means of inversion. Immediately prior to use, Silwett L-77 was added to a final concentration of 0.02% (v/v) and the solution mixed well. *Arabidopsis thaliana* plants with immature floral buds were inverted and the aerial parts up to the rosette were dipped in the *Agrobacterium* cell suspension for 10 s with gentle agitation. Dipped plants were removed from the solution and allowed to drain for 3-5 s. The treated plants were sealed in plastic bags and laid down horizontally and kept in the dark for 16-24 h, after which the plastic was removed and the plants were sent back to the greenhouse to grow normally. After seven days the floral dip method was repeated with the same constructs to increase the transformation efficiency. For the double transformation event, the resuspended *Agrobacterium* cells of the two plasmids were combined in the sucrose solution before floral dipping the *Arabidopsis* plants. Dry seeds were collected once the siliques had turned brown and opened. Seeds were stored in open microfuge tubes in a sealed container filled with silica gel at 4°C.

2.16 Growing transgenic *Arabidopsis thaliana* for seed production

Putatively transformed *A. thaliana* T₁ seeds were surface decontaminated, allowed to germinate on growth media and grown under tissue culture growth conditions as previously described with the exception that the media contained 100 mg/L carbenicillin to kill the *Agrobacterium* and, 50 mg/L kanamycin and/or 25 mg/L hygromycin (depending on which plasmid was used to transform) for selection of transformants. After 14 d on the selective medium the putative positive transformants were carefully removed from the plates and transferred to fresh selection plates and grown for another two weeks before being transplanted in water-saturated Jiffy-7[®] bags (Jiffy Products Int.),

which consist of compressed peat held together by fine netting. The Jiffy-7[®] bags were placed in a plastic tray and covered with plastic film for two days to maintain a high humidity, after which the plantlets were hardened-off and grown for three to four weeks, to establish a strong vegetative state, in a 10h:14h light:dark photoperiod, with a light intensity of 90 $\mu\text{moles photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at a 21°C:15°C day:night temperature range with humidity averaging 90%. The plants were watered by sub-irrigation every three to four days. Any inflorescences that emerged in this time period were trimmed down near the base of the rosette.

Once the plants had achieved a strong vegetative state, they were moved to a greenhouse with a longer day length to initiate bolting. After the siliques turned brown and completely dried, the T₂ seeds were harvested. These T₂ generation seeds were treated the same way as the T₁ seeds with the exception that the tissue culture media did not contain carbenicillin. The different transgenic lines were tested for gene insertion and expression via PCR (see section 2.18 and 2.21 respectively). Those which did not pass were omitted from further analysis and seed production. The positive transgenic lines were grown until seed formation and dried to collect the T₃ seeds. Further experiments were carried out with the T₃ generation unless stated otherwise. The T₃ transgenic lines were planted in Jiffy-7[®] bags and also tested for gene insertion and expression.

2.17 Genomic DNA extraction from plant tissue

Genomic DNA was isolated from *A. thaliana* using a protocol from McGarvey and Kaper (1991). One leaf was frozen in a 1.5 mL micro-centrifuge tube with liquid nitrogen and ground to a fine powder using a small plastic pestle that fits inside the micro-centrifuge tube. After grinding, 400 μL extraction buffer (1% [m/v] cetyltrimethylammonium [CTAB], 50 mM Tris-HCl pH 8.0, 0.7 M NaCl, 10 mM EDTA pH 8.0, 0.5% [m/v] polyvinylpyrrolidone [PVP], 0.1% [v/v] β -mercaptoethanol) was added to the tube and vortexed for 10 s. After incubation for 1 h at 60°C, 400 μL chloroform:isoamyl alcohol (24:1) was added to the tube, the sample quickly vortexed at top speed and centrifuged for 5 min at 16 000 xg at room temperature. The supernatant was transferred to a new 1.5 mL micro-centrifuge tube and one volume of ice-cold isopropanol was added and incubated at -20°C for 15 min to precipitate the DNA. The tube was centrifuged at 16 000 xg for 10 min at 4°C. The resulting pellet was washed with 70% (v/v) ethanol and centrifuged at 16 000 xg for 2 min at room temperature. The supernatant was discarded before the pellet was dried in a laminar flow cabinet for 30 to 60 min and resuspended in 20 μL ddH₂O. Isolated gDNA was quantified by a NanoDrop ND 1000 (Thermo Fisher Scientific) spectrophotometer and evaluated on a 1% agarose gel stained with ethidium bromide before being stored at -20°C until use.

2.18 Genomic DNA PCR

Putative *ALDC* transgenic *Arabidopsis* lines were screened, after kanamycin selection, using *ALDC_FW* and *ALDC_RW* primers (Table 2.1). Putative *BDH1* transgenic lines were screened, after hygromycin selection, using *BDH1_FW* and *BDH1_RW* primers (Table 2.1). Putative double transgenic lines *ALDC/BDH1* were screened, after selection on both hygromycin and kanamycin, using both primer sets (Table 2.1). Amplification was performed using the GoTaq™ DNA Polymerase kit (Promega) with reactions set up as described in Table 2.3. The recommended cycling protocol for GoTaq™ was used in a T100™ Thermal Cycler (Bio-Rad) under the conditions described in Table 2.4. DNA fragments were separated by gel electrophoresis and viewed under UV-light as previously described.

Table 2.3: Reagents and volumes used for PCR amplification of the *ALDC* and *BDH1* genes.

Component	Final volume
5X Green GoTaq™ Reaction Buffer	10 µL
dNTP Mix, 10 mM each	1.5 µL
Upstream Primer, 10 mM	1.5 µL
Downstream Primer, 10 mM	1.5 µL
GoTaq™ DNA Polymerase (5 U/µL)	0.1 µL
Template DNA (<0.5 µg/50 µL)	2.0 µL
Nuclease-Free Water to	50 µL

Table 2.4: PCR cycling protocol for GoTaq™ of the *ALDC* and *BDH1* genes.

PCR step	Cycling conditions		No. of cycles
	Temperature	Time	
Initial denature	95°C	2 min	1
Denature	95°C	1 min	
Annealing (T _m)	65°C	1 min	35
Extension	72°C	1 min	
Final elongation	72°C	10 min	1

2.19 Total RNA extraction from plant tissue

Total RNA was extracted from *Arabidopsis* leaf material using a CTAB extraction protocol. One leaf was frozen in a 1.5 mL micro-centrifuge tube with liquid nitrogen and ground to a fine powder

using a small plastic pestle that fits inside the micro-centrifuge tube. The frozen powder was transferred to a 2 mL micro-centrifuge tube and 1.5 mL of extraction buffer (2% [m/v] CTAB, 2% [m/v] PVP, 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 2 M NaCl, 2% [v/v] β -mercaptoethanol [added immediately prior to extraction]) was added to the powder before vortexing at maximum speed for 30 s. After incubation at 65°C for 30 min with vortexing every 5 min the tube was centrifuged for 10 min at 16 000 xg at room temperature. Next, 950 μ L of the supernatant was transferred to a new 2 mL micro-centrifuge tube. One volume chloroform:isoamyl alcohol (24:1) was added to the tube and vortexed for 30 s after which it was centrifuged for 10 min at 16 000 xg at 4°C. The supernatant was transferred to a new micro-centrifuge tube and the chloroform:isoamyl extraction was repeated. The supernatant was again transferred to a new 1.5 mL micro-centrifuge tube with the addition of LiCl to a final concentration of 2 M and incubated overnight at -20°C to precipitate the RNA. The sample was centrifuged for 60 min at 16 000 xg at 4°C to pellet the RNA. The supernatant was discarded and the pellet washed with 70% ethanol. The ethanol was decanted and the sample centrifuged at 16 000 xg for another 20 min at 4°C. The residual ethanol was removed and the pellet air dried in a laminar flow for 30 min and then resuspended in 30 μ L nuclease-free ddH₂O. The RNA concentration was quantified via a NanoDrop ND 1000 (Thermo Fisher Scientific) spectrophotometer before being treated with DNase (DNase I, RNase-free; Fermentas) according to the manufacturer's recommendations. DNase-free RNA samples (5 μ L) were run on a 2% (m/v) agarose gel and viewed under UV-light as previously described to determine the integrity. The remaining DNase-free RNA samples were stored at -80°C.

2.20 Complementary DNA synthesis

Complementary DNA was synthesized as per manufacturer's recommendation using the Maxima H minus first strand cDNA synthesis kit (Thermo Scientific) using 1 μ g of previously DNase-treated RNA and using oligo (dT)₁₈ primers as specified. Complementary DNA was stored at -80°C.

2.21 Semi-Quantitative PCR

Two μ L of cDNA was used in 50 μ L PCR reactions as previously described for gDNA PCR to determine gene expression, with the exception that 25 cycles were used instead of 35 for denaturing, annealing and extension. The constitutively expressed *actin2* gene was amplified using the primers indicated in Table 2.5 and used as a positive control. *ACT2* primers were tested for cycle linearity by performing a PCR with *A. thaliana* cDNA as template. Various cycles were terminated by adding 5 μ L 500 mM EDTA to the PCR tube. After evaluation of the PCR products on a 1 % agarose gel, cycle 25 was determined to be the cycle where linearity was reached.

Table 2.5: Primers designed and used in PCR amplification to amplify the *Actin2* gene.

Primer name	Sequence	Product Size
ACT2_FW	5'-GATATGGAAAAGATCTGGCATCAC-3'	1000 bp
ACT2_RW	5'-TCATACTCGGCCTTGGAGATCCAC-3'	

2.22 Amino acid analysis

Arabidopsis thaliana seeds were placed on moist Jiffy-7[®] bags and kept in the dark at 4°C for 3 days before being moved to a growth room with a 10h:14h light:dark photoperiod, with a light intensity of 90 $\mu\text{moles photons.m}^2.\text{s}^{-1}$ at 21°C:15°C day:night temperature range with humidity averaging 90 percent. Seven and 21 d post-germination each Jiffy-7[®] bag was watered with 10 mL 1.4 g/L All Purpose Plant Food (Phostrogen, UK). Plants were watered by sub-irrigation every 3 to 4 d.

Free amino acids were extracted from 8-week-old *Arabidopsis* plants using a protocol from Hacham et al. (2002). Leaves were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. Approximately 200 mg of frozen powdered leaf material was homogenized with 600 μL water:chloroform:methanol (3:5:12 [v/v]) by vortex for 30 s in a 2 mL micro-centrifuge tube. The tube was centrifuged for 1 min at 16 000 xg at room temperature. The supernatant was transferred to a new 2 mL micro-centrifuge tube and the water:chloroform:methanol extraction of the tissue was repeated. The supernatant was added to the first supernatant and 300 μL chloroform and 450 μL water were added. The mixture was vortexed for 30 s and centrifuged at room temperature for 5 min at 16 000 xg . The upper water-methanol phase was transferred to a new 1.5 mL micro-centrifuge tube and dried in a GeneVac[®] EZ-2 personal evaporator (SP Scientific) overnight. The dried pellet was dissolved in 200 μL 20 mM HCl.

Samples were derivatised, using the AccQ Tag Ultra Derivatization Kit (Waters, <http://www.waters.com>), by adding 10 μL from each undiluted sample to the kit constituents and placing them in a heating block at 55°C for 10 min. The liquid chromatography-mass spectrometry (LC-MS) analysis for amino acids was performed by the Central Analytical Facility (CAF) at Stellenbosch University on a Waters API Quattro Micro (Milford, MA, USA) instrument and linked to a Waters Acquity ultra-performance chromatograph (UPLC). Positive ionization was achieved with electrospray ionization (ESI), capillary voltage 3.5 kV and cone voltage of 15 V. Nitrogen was used as the desolvation gas at 350°C with a flow rate of 350 L/h. One μL was injected into a Waters AccQ tag C18 column (1.7 μm particle size, 2.1 x 100 mm). The derivatives were eluted with dilutions of Waters AccQ Tag Ultra Eluent A and B prior to UV detection for quantification. A gradient was set up and initiated by combining 99.9% eluent A and 0.1% eluent B (holding time of 0.54 min). A linear gradient to 21.2% eluent B over 7.2 min, to 90% eluent B over 0.31 min, to 100% eluent B over 0.45 min then followed. The column was kept at 100% eluent B for another 1

min to yield a total run time of 9.5 min. The flow rate was maintained at 0.7 mL/min throughout the run.

2.23 Exogenous application of synthetic acetoin and 2,3-butanediol

Synthetic standards of acetoin (Sigma-Aldrich) and 2,3-butanediol (Fluka) were tested against *Arabidopsis* seedlings using a modified method from Ryu et al. (2003). *Arabidopsis* seeds were treated, plated (with the exception of using 1.5% sucrose instead of 3%; Fig 2.2) and grown horizontally as previously described. The synthetic standard (acetoin or 2,3-butanediol) diluted in dichloromethane (DCM) resulting in various concentrations (20 µg, 200 ng, 2 ng, 20 pg), or DCM alone was mixed with 80 mg lanolin wax. Next, 20 µL of the suspension was applied onto a glass cover slip (Fig 2.4) at the two day-old germinated seedling stage inside the culture dish. Plates were sealed with surgical tape (3M Micropore™) and grown under tissue culture growth conditions as previously described. A second experiment was conducted under shorter day length conditions, using a 10h:14h light:dark photoperiod, under cool, white fluorescent tubes (Osram L 58V/740) with a light intensity of 50 µmoles photons.m⁻².s⁻¹ at 22°C in a growth cabinet (Snijders Scientific). Ten days after addition of the synthetic standards, total leaf surface area were digitally acquired by a Canon EOS 500D camera and analyzed by imaging software by comparing total leaf area with a 1 cm² reference marker.

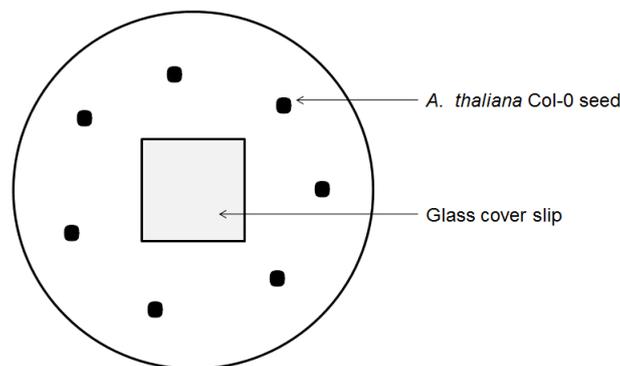


Figure 2.2: Plate layout of synthetic VOC experiment.

2.24 Growth comparison between wild type and transgenic *Arabidopsis* plants in tissue culture conditions

Arabidopsis thaliana seeds were surface decontaminated, allowed to germinate on growth media and grown vertically under tissue culture growth conditions as previously described. For selection

type experiments the transgenic lines were germinated and grown for 5 d, post-germination, on selection media, containing kanamycin and/or hygromycin, before being carefully transferred to normal fresh growth media, unless stated otherwise. All data were collected 14 d post germination. Each experiment consisted of 10 replicates of five seeds per plate and was repeated 3 times independently, unless stated otherwise.

After the growth period the plants leaf area was digitally acquired as previously described after which the plants were carefully removed from the plates and blotted dry before being measured for whole fresh mass. To obtain dry mass, all 5 whole plants per plate were pooled before drying for 3 days at 60°C.

2.25 Plant crude extracts

Plant crude extracts were prepared as in Peters et al. (2007). Freshly harvested leaf material (200 mg) was cut into small pieces with a sterile scalpel blade and ground in 800 µL ice-cold extraction buffer (50 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES]/KOH pH 7.5, 5 mM MgCl₂, 20 mM dithiothreitol [DTT], 1 mM EDTA, 2% [w/v] PVP K30, 0.1% [v/v] Triton X-100, 1 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride [PMSF], 50 mM Na ascorbate) to a fine paste. The samples were centrifuged at 14 000 *xg* for 10 min at 4°C. The supernatant was removed and desalted using a molecular size exclusion column of 3 kDa (Amicon[®] Ultra Centrifugal Filter Units) before being used for enzymatic assays.

2.26 A quantitative assay for BDH1 activity

A quantitative assay for the detection of BDH1 activity was based on a method described by Nicholson (2008) using the reaction: acetoin + NADH → 2,3-butanediol + NAD⁺ to monitor the acetoin-dependent consumption of NADH. Cell extracts (untransformed control *A. thaliana* and *BDH1* transformed plants) were prepared as previously described and assayed for BDH1 activity based on a method for assaying glucose dehydrogenase activity (Fujiya et al., 1977). Briefly, 50 µL cell extract was added to 200 µL assay buffer (500 mM Tris-HCl pH 7.5, 1.2 M KCl, 1 mM EDTA, 50 mM acetoin, 0.2 mM NADH) at 30°C. The oxidation of NADH to NAD⁺ was monitored at A_{340 nm}.

2.27 Enzymatic assay for ALDC activity

An enzymatic assay for the quantification of ALDC activity was performed as described in www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/a-acetolactate_decarboxylase.pdf using untransformed control *A. thaliana* and ALDC transformed

plants. Briefly, 200 μL α -acetolactate was added to 200 μL cell extract (as previously described) and incubated for 20 min at 30°C before adding 4.5 mL colour reagent (1% α -naphthol, 0.1% creatine, 1 M NaOH) and incubated at room temperature for 40 min. The formation of acetoin from α -acetolactate by α -acetolactate decarboxylase was monitored at $A_{522 \text{ nm}}$.

2.28 Gas chromatography-mass spectrometry (GC-MS) detection of leaf volatiles

Note: Following sample preparation, the GC-MS analysis was conducted by Prof B Burger (Department of Chemistry and Polymer Science, Stellenbosch University)

Gas chromatography-mass spectrometry detection of leaf volatiles were based on a method by Burger et al., 2011. Whole fresh leaves (300 mg) of *ALDC* transgenic *Arabidopsis* plants were harvested immediately prior to use and/or ground in liquid nitrogen before being transferred to a 50 mL glass bottle. All traces of adsorbed organics were removed from the glassware, by thorough washing with ddH₂O prior to heating at 400°C for at least 30 min, and then cooled before use. A second generation SEP30 sample enrichment probe (SEP, [MasChrom Analisetegniek, Stellenbosch, South Africa]), consisting of a fused silica stalk, or shaft, carrying a 30 mm sleeve of polydimethylsiloxane (PDMS) tubing (Burger et al., 2006; Burger et al., 2011), was used to trap VOCs from leaf samples for GC-MS analyses. The PDMS sleeve of the SEP30 was exposed to the headspace volatiles of the leaf samples at 22°C for 24 h after which the volatile compounds retained in the PDMS were desorbed at 220°C in the GC-MS. The SEP was left in the injector until completion of an analysis (Burger et al., 2006).

Gas chromatographic-low resolution electron impact mass spectrometric analysis (GC-LRMS) of *ALDC* transgenic *Arabidopsis* leaf samples was carried out on a Finnigan MD800 instrument, using a 30 m x 0.25 mm i.d. Zebron™ ZB-5MS column (Phenomenex®). The column was programmed at 4°C/min from 30°C to 200°C. Electron impact (EI) mass spectral data were acquired at 70 eV from m/z 25-150. Helium was the carrier gas at a linear flow velocity of 28.6 $\text{cm}\cdot\text{s}^{-1}$ measured at a column temperature of 40°C. Inlet and interface temperatures were 220°C and 250°C, respectively. The ion source temperature was set at 180°C, and the pressure in the source housing was $ca. 2 \times 10^{-5}$ mm Hg at a column temperature of 40°C. A scan rate of 0.9 $\text{s}\cdot\text{scan}^{-1}$ and an interscan delay of 0.1 s were used. This instrumentation was also used for retention time comparisons of the reference standards with authentic synthetic reference compounds.

2.29 High-performance liquid chromatography (HPLC) analysis

Note: Following sample preparation, the HPLC analysis was conducted by Dr H Eyeghe-Bickong (Institute for Wine Biotechnology, Stellenbosch University)

High-performance liquid chromatography (HPLC) analysis was performed on untransformed control *A. thaliana* and transformed plants based on previous papers (Zeppa et al., 2001; Nicholson, 2008; Eyéghé-Bickong et al., 2012). Briefly, 2 week old tissue culture seedlings were ground up in liquid nitrogen to a fine powder, 200 mg of the frozen homogenized leaf tissue was added to 500 μ L dH₂O (containing 1 g/L adipic acid and ribitol [as internal standards [IS] for organic acids and sugars respectively]) and vortexed for 1 min after which it was centrifuged at 13 000 xg for 15 min at 4°C. The supernatant was filtered through a 0.22 μ m nylon filter before HPLC analysis. Acetoin (5 g/L) and 2,3-butanediol (5 g/L) standards were also prepared in dH₂O and filtered before HPLC analysis. Sample components (injection volume of 20 μ L) were determined using an Agilent 1100 series HPLC system (Agilent Technologies) equipped with a diode array detector (DAD) coupled to a refractive index detector (RID). The system used an Aminex HPX-87H ion exchange column (300 mm x 7.78 mm, Bio-Rad Laboratories) protected with a Bio-Rad guard cartridge (20 mm x 4.6 mm). The column temperature was 45°C, with a flow rate 0.4 ml/min 5 mM H₂SO₄. ChemStation software (Agilent Technologies) was used to process the chromatograms. Acetoin (as well as organic acids) were detected with the DAD at 210 nm but with less noise (no organic acids) at 278 nm, whilst 2,3-butanediol (as well as sugars) were detected with the RID.

2.30 Statistical analyses

Unless otherwise stated, statistical analyses were performed using the t-test embedded in Microsoft Excel. *P* values ≤ 0.05 were considered to be statistically significantly different from the control samples.

3. Results and Discussion

3.1 Construction of the plant transformation vectors

Volatiles released from *Bacillus subtilis* GB03 showed a growth promoting effect on *A. thaliana* with the major volatiles being acetoin and 2,3-butanediol (Ryu et al., 2003). The α -acetolactate decarboxylase gene is responsible for the conversion of acetolactate to acetoin followed by acetoin reductase/2,3-butanediol dehydrogenase to form 2,3-butanediol (Ryu et al., 2003). Plant transformation vectors containing the genes for α -acetolactate decarboxylase and acetoin reductase/2,3-butanediol dehydrogenase were constructed and transformed into *A. thaliana* in an attempt to express the respective genes either alone or together.

To isolate an α -acetolactate decarboxylase gene, various α -acetolactate decarboxylase genes were compared to identify a conserved amino acid sequence, but no definitive conserved region could be identified among Gram-positive sequences (data not shown). Gram-positive bacteria also have a different codon usage than eukaryotes and gene expression could be greatly impaired due to inappropriate amino acid substitutions, AT-rich localized regions or the presence of messenger RNA-destabilizing sequences (Röber et al., 1996; Ye et al., 2001). To avoid such problems, Röber et al. (1996) suggested that genes from Gram-negative organisms should improve gene expression in plants compared to that of Gram-positive genes. Gram-negative sequences were then compared and showed more highly conserved regions compared with Gram-positive sequences (data not shown), however, most of the species were pathogenic to humans or plants. Iglesias et al. (1997) also reported that reduced transgene expression is observed in plants and mammals when using sequences from bacterial origin. Thus, to avoid any such problems, it was decided to search for conserved regions in eukaryotic species as it would be easier to work with these genes in plants. *Aspergillus* species are fairly common and *Aspergillus niger* ATCC 10864 (due to availability) was chosen for isolation of the α -acetolactate decarboxylase gene (An03g00490 from AspGD). For the same reasons, *S. cerevisiae* W303 was chosen for isolation of the acetoin reductase/2,3-butanediol dehydrogenase gene (YAL060W from SGD).

In plants, α -acetolactate is located in the chloroplast (Mifflin, 1974), therefore the plant transformation vectors used (pCambia2300::*FNR* and pCambia1300::*FNR*, kind gift by Dr Lloyd, Stellenbosch) contained the chloroplast localization peptide sequence from ferredoxin-NADP⁺ reductase (*FNR*, EC 1.18.1.2) from *Spinacia oleracea*. Primers (Table 2.1) were designed, with additional restriction sites at the ends, to amplify the 960 bp *ALDC* gene, responsible for acetoin formation from *A. niger* and the 1149 bp *BDH1* gene, responsible for 2,3-butanediol production from acetoin, from *S. cerevisiae*. After PCR cleanup, the respective genes were blunt ligated into the pJet1.2 vector (Fig 2.1) and then transformed into *E. coli* DH5 α competent cells. Various colony PCRs were performed on the transformed cells to confirm insertion and the positive colonies were subjected to plasmid isolation. The resulting plasmids were bidirectionally

sequenced to confirm the identity of the insert. Sequence data for the *ALDC* (Fig 3.1) and *BDH1* (Fig 3.2) genes thus isolated were aligned against their respective genes of origin. The respective plasmids with no errors in the gene sequences were double digested, using the relevant restriction enzymes designed into the primer sequences used to amplify the genes, to isolate the *ALDC* or *BDH1* genes before these were ligated into the pCambia2300::*FNR* and pCambia1300::*FNR* plasmids, respectively (Fig 3.3; Fig 3.4). Although a second *SaI* site was present in the pCambia1300::*FNR* vector, partial digestion with *SaI* was carried out on the vector, after full digestion with *SacI*, before being subjected to agarose gel analysis. The correct digestion product was identified on the basis that no ~200 bp band was digested from the vector (Fig 3.4).

Agrobacterium tumefaciens GV3101 competent cells were transformed with pCambia2300::*FNR:ALDC* (Fig 3.3) and pCambia1300::*FNR:BDH1* (Fig 3.4). Various colony PCRs were performed to confirm insertion on the transformed cells. The positive colonies were then used to prepare glycerol stocks for long-term storage.

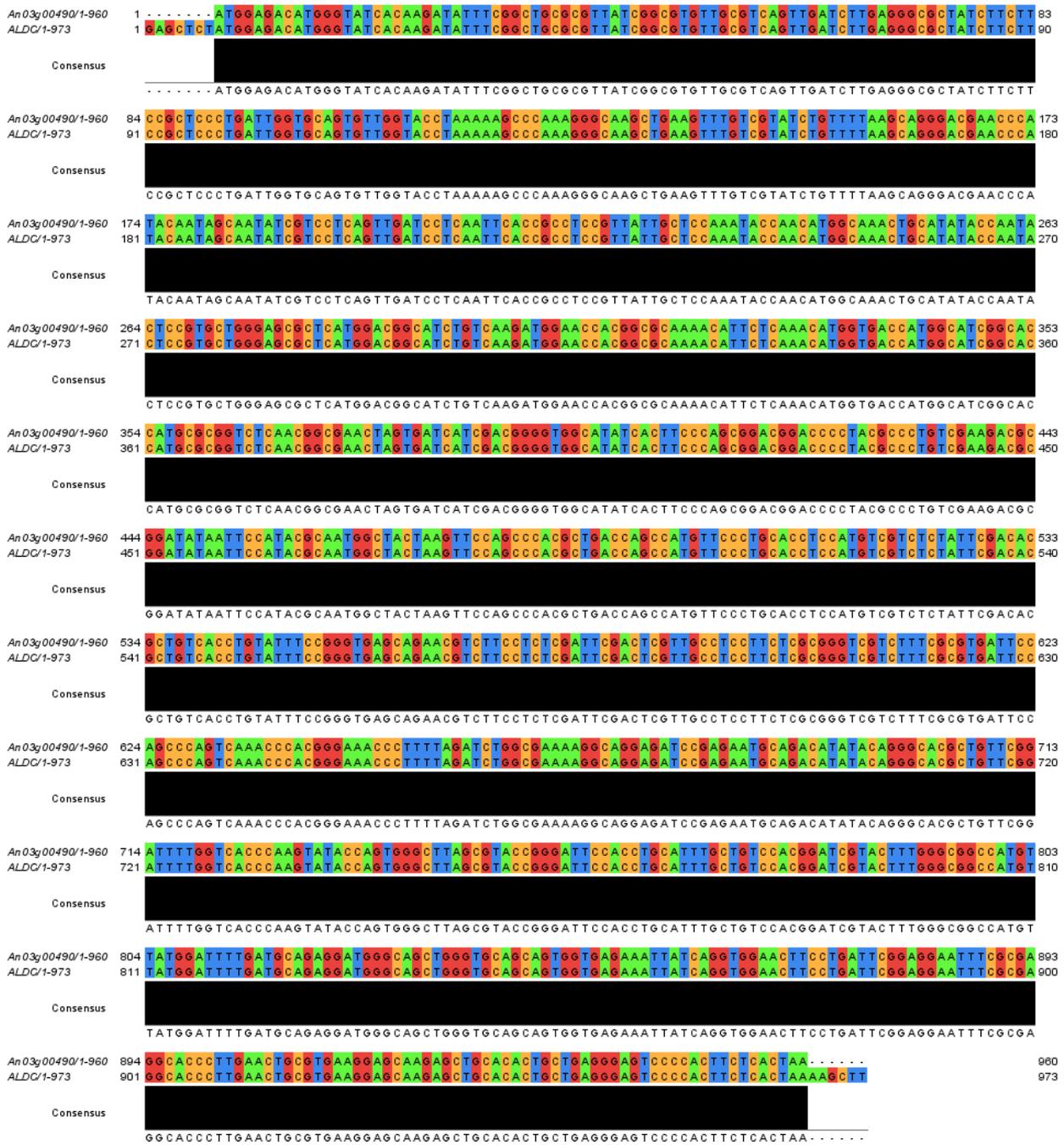


Figure 3.1: Alignment of *Aspergillus niger* An03g00490 (labeled *An03g00490/1-960*) from the *Aspergillus* Genome Database with the α -acetolactate decarboxylase insert (labeled *ALDC/1-973*) from the pJet1.2::*ALDC* vector that was sequenced using the pJet1.2 forward and reverse primers. Sequences were edited to remove vector sequences and then aligned using ClustalW (www.genome.jp/tools/clustalw) and displayed in JalView (www.jalview.org). Non-conserved overhangs represent restriction sites incorporated into the gene for cloning purposes.

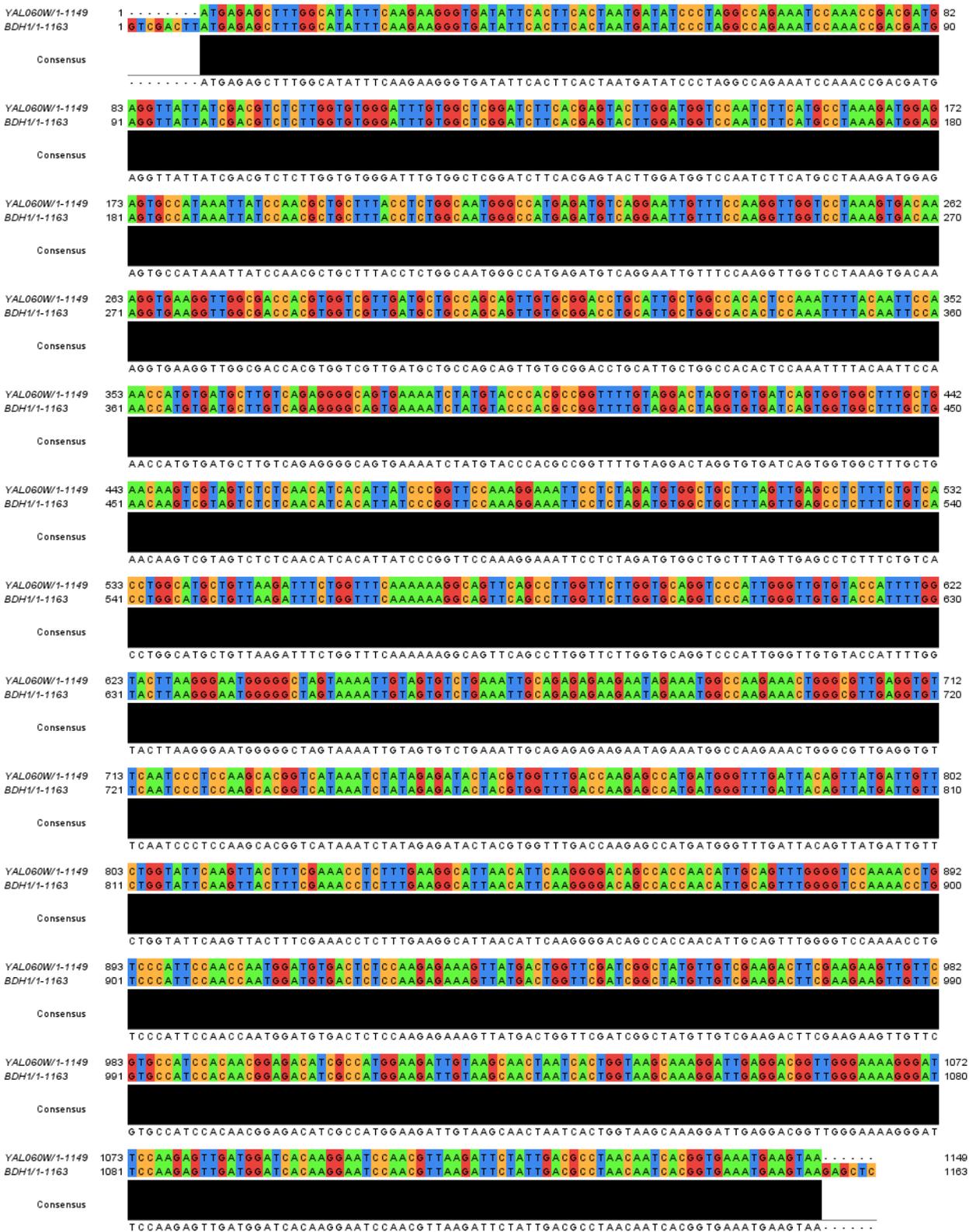


Figure 3.2: Alignment of *Saccharomyces cerevisiae* YAL060W (labeled YAL060W/1-1149) from the *Saccharomyces* Genome Database with the acetoin reductase/2,3-butanediol dehydrogenase insert (labeled BDH1/1-1163) from pJet1.2::BDH1 vector that was sequenced using the pJet1.2 forward and reverse primers. Sequences were edited to remove vector sequences and then aligned using ClustalW (www.genome.jp/tools/clustalw) and displayed in JalView (www.jalview.org). Non-conserved overhangs represent restriction sites incorporated into the gene for cloning purposes.

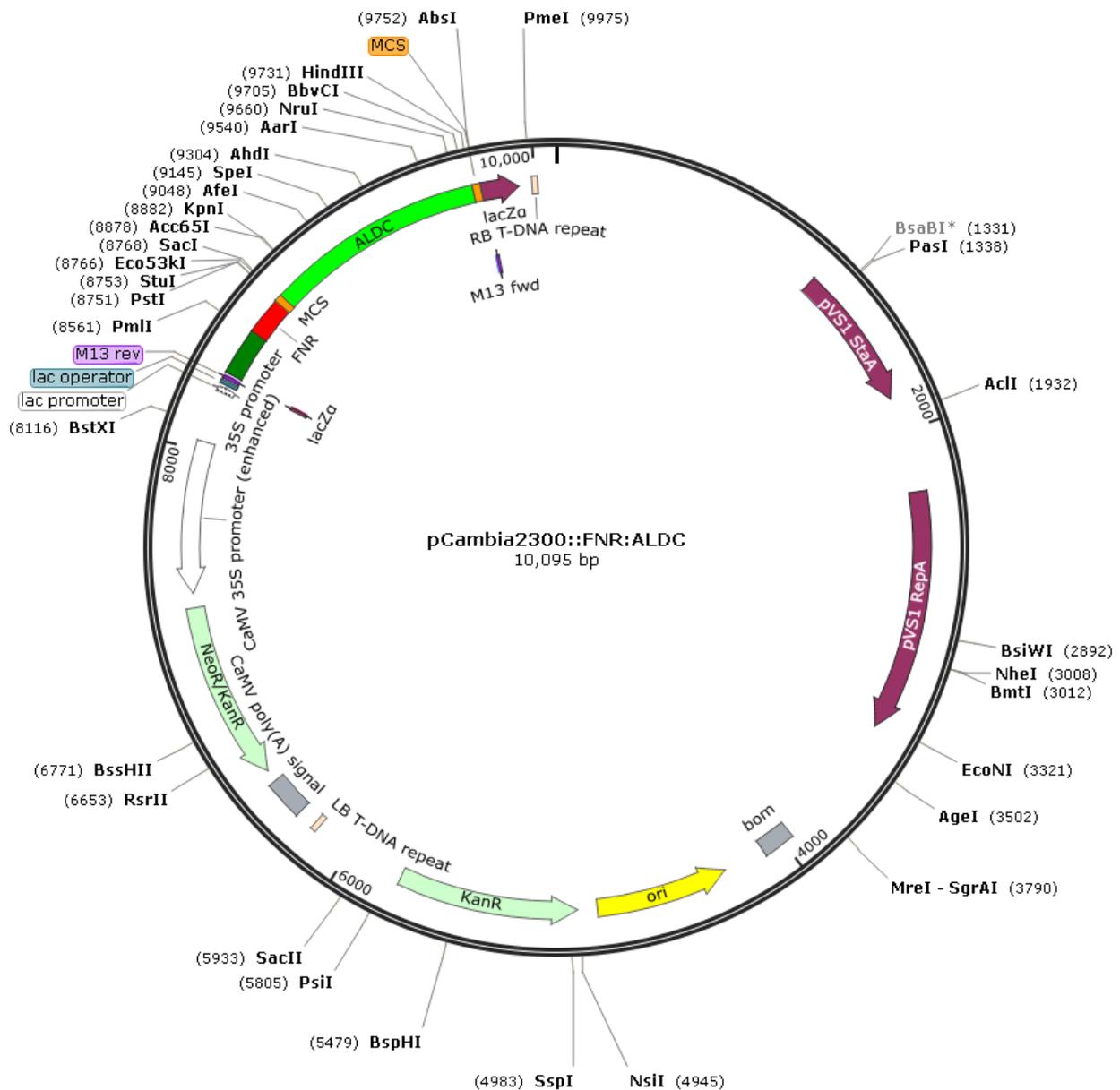


Figure 3.3: Plasmid map of constructed plant transformation vector pCambia2300::FNR:ALDC. The pCambia2300 plant transformation vector was altered by taking an *EcoRI/HindIII* fragment from pBINAR containing the CaMV35S promoter, polylinker with the ferredoxin-NADP⁺ reductase (FNR) chloroplast transit peptide sequence and terminator, and ligating that into the *EcoRI/HindIII* sites of pCambia2300. The α -acetolactate decarboxylase (ALDC) gene was then ligated in-frame with the FNR sequence using the *SacI* and *HindIII* restriction sequences designed into the primers used to amplify the gene.

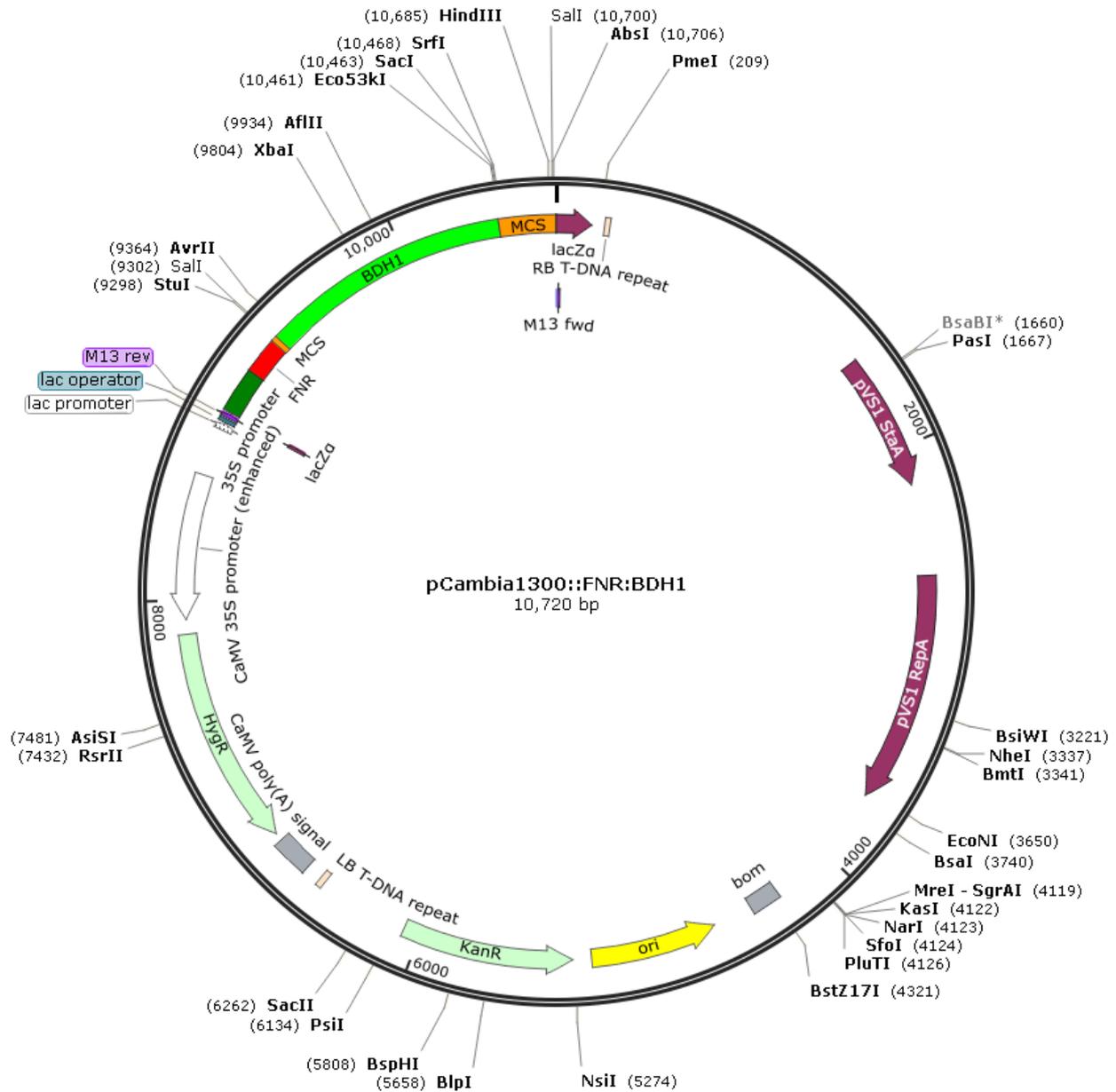


Figure 3.4: Plasmid map of constructed plant transformation vector pCambia1300::FNR:BDH1. The pCambia1300 plant transformation vector was altered by taking an *EcoRI/HindIII* fragment from pBINAR containing the CaMV35S promoter, polylinker with the ferredoxin-NADP⁺ reductase (FNR) chloroplast transit peptide sequence and terminator and ligating that into the *EcoRI/HindIII* sites of pCambia1300. The acetoin reductase/2,3-butanediol dehydrogenase (*BDH1*) gene was then ligated in-frame with the FNR sequence using the *SacI* and *HindIII* restriction sequences designed into the primers used to amplify the gene.

3.2 Transformations and selection

Arabidopsis thaliana Col-0 plants were transformed by means of *Agrobacterium*-mediated floral dipping using the pCambia2300::*FNR:ALDC* and/or pCambia1300::*FNR:BDH1* vectors (Fig 3.3; Fig 3.4). The T₁ seeds were germinated and grown in tissue culture on selection media containing either 50 µg/mL kanamycin for pCambia2300::*FNR:ALDC*, 25 µg/mL hygromycin for pCambia1300::*FNR:BDH1* or both antibiotics for the double transformation using both plasmids, for a total of four weeks. Putative transformants were identified by the ability to survive the antibiotic screening (Fig 3.5). As expected, the number of putative double transformants was much lower (Fig 3.5 C) than either of the putative single transformants (Fig 3.5 A,B).

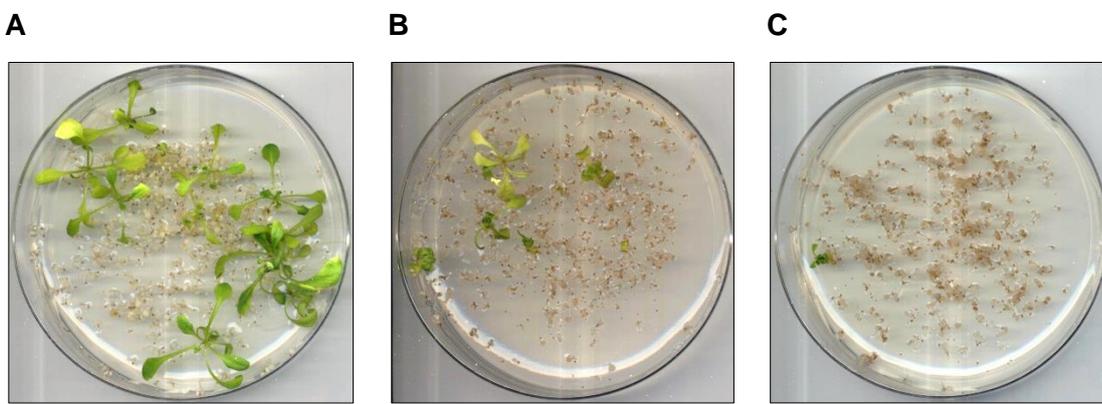


Figure 3.5: Antibiotic selection of transgenic *Arabidopsis thaliana* plants putatively transformed with **A**) the α -acetolactate decarboxylase (*ALDC*) gene from *Aspergillus niger*, **B**) the acetoin reductase/2,3-butanediol dehydrogenase (*BDH1*) gene from *Saccharomyces cerevisiae*, **C**) both the *ALDC* and *BDH1* genes.

After antibiotic screening plantlets were grown in Jiffy-7[®] bags and allowed to produce seed. The T₂ seeds were germinated and grown in tissue culture on selection media for two weeks before being transferred to Jiffy-7[®] bags. Once the plants had achieved a strong vegetative state, gDNA was extracted from leaf material. True transformants were identified by PCR using gene specific primers (Table 2.1). Several positive transgenic lines were identified (Fig 3.6). Only one of the *ALDC* lines (A7) was a false positive (Fig 3.6 A), whilst all the lines of the *BDH1* transformants were positive (Fig 3.6 B). One of the putative double transformants, line AB5, contained only the *BDH1* transgene, whilst the other putative double transformed lines tested positive for both transgenes (Fig 3.6 C). All the false positive lines were rejected from any further studies. After confirmation of transgene insertion, one or two leaves were harvested, flash frozen and stored at -80°C for gene expression analysis. All of the positively confirmed insertion lines were allowed to seed.

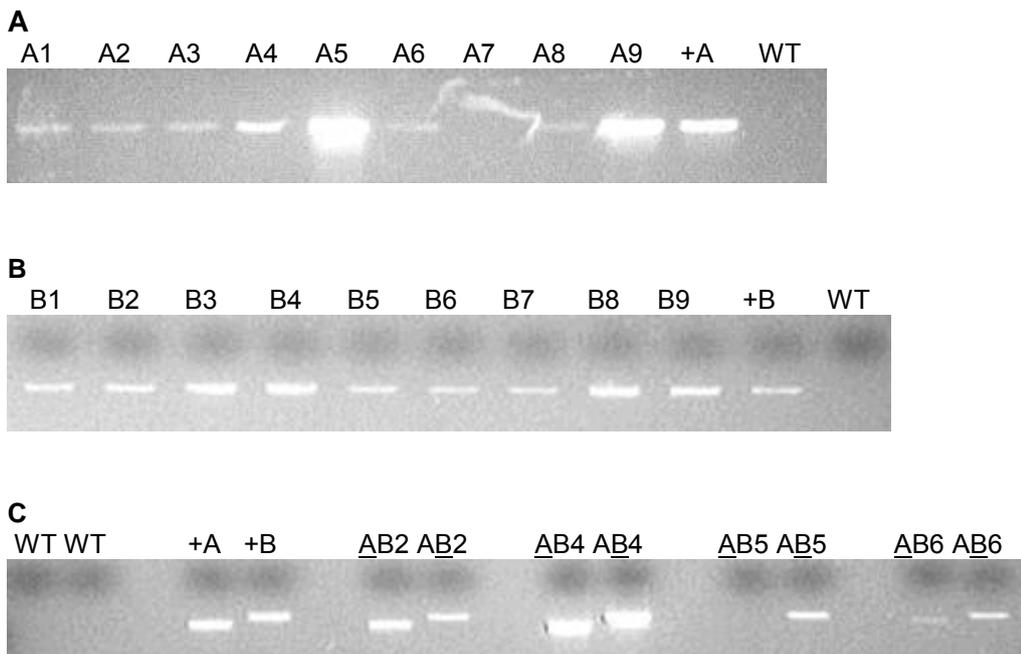


Figure 3.6: PCR screening of T_2 transgenic *A. thaliana* lines transformed with **A)** the α -acetolactate decarboxylase (*ALDC*) gene from *A. niger*, **B)** the acetoin reductase/2,3-butanediol dehydrogenase (*BDH1*) gene from *S. cerevisiae*, and **C)** both the *ALDC* and *BDH1* genes. WT: *A. thaliana* Col-0; +A: pCambia2300::*FNR:ALDC*; +B: pCambia1300::*FNR:BDH1*; A1-9: Lines from T_2 generation transgenic plants transformed with pCambia2300::*FNR:ALDC*; B1-9: Lines from T_2 generation transgenic plants transformed with pCambia1300::*FNR:BDH1*; AB2-6: Lines from T_2 generation double transgenic plants transformed with pCambia2300::*FNR:ALDC* and pCambia1300::*FNR:BDH1*.

Before seeding the T_3 seed, *ALDC* and *BDH1* transcript levels in the stored leaf material from the respective T_2 plants were measured by semi-quantitative RT-PCR (sqRT-PCR) in comparison to the constitutively expressed *ACT2* gene. Several *ALDC* lines showed expression, however, lines A2 and A8 were rejected from further studies as they showed no expression. Lines A4, A5 and A9 had very weak expression compared to lines A1, A3 and A6 which showed high levels of expression (Fig 3.7 A). With regard to the *BDH1* transformants, line B3 showed no expression, lines B5 and B9 had very low expression levels, lines B1, B4 and B8 showed medium expression and lines B2, B6 and B7 had high levels of expression (Fig 3.7 B). No gene expression data could be obtained for the T_2 double transformants due to the material being lost in a -80°C freezer malfunction shortly after the single gene insertion lines were tested for gene expression.

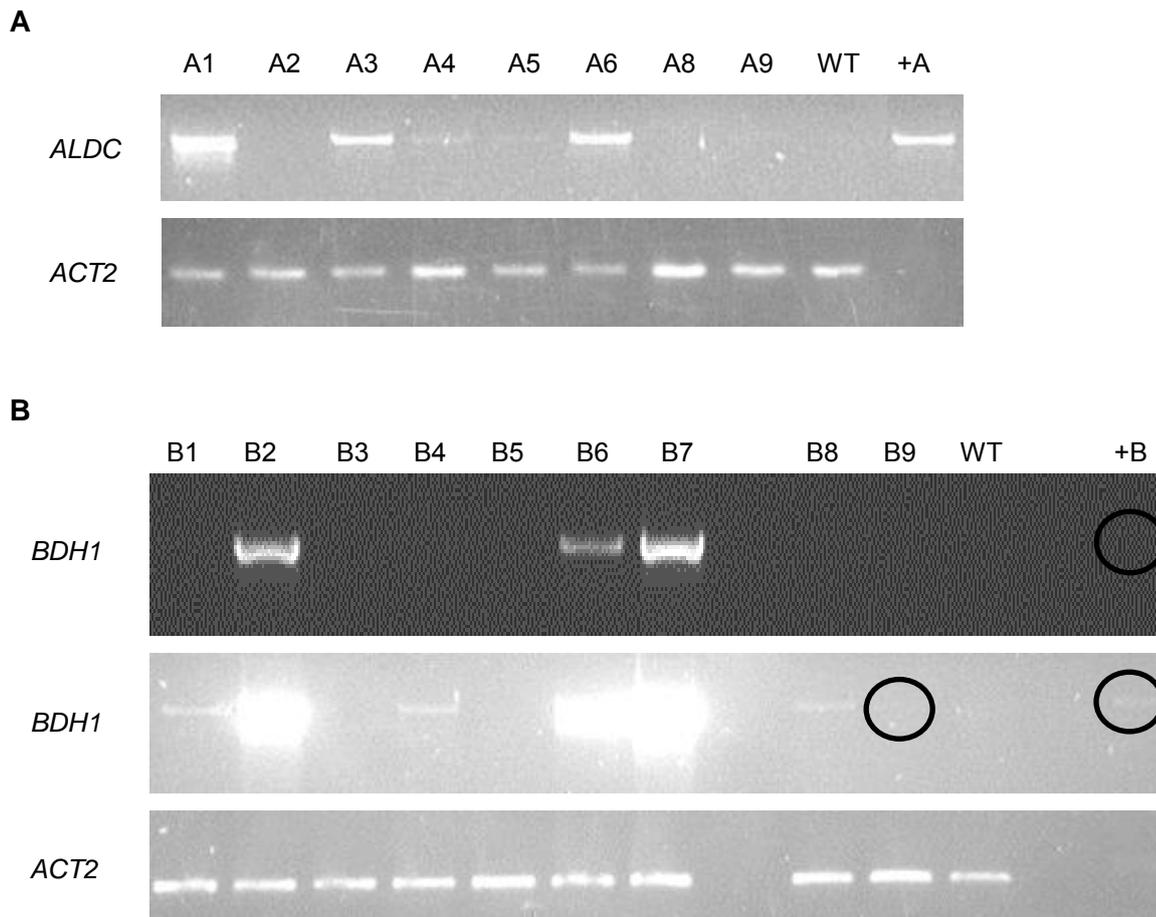


Figure 3.7: Semi-quantitative RT-PCR showing transcription of the **A)** α -acetolactate decarboxylase (*ALDC*) gene and **B)** acetoin reductase/2,3-butanediol dehydrogenase (*BDH1*) gene [top *BDH1*: normal exposure, bottom *BDH1*: overexposed to visualize the fainter bands] in T₂ transgenic *A. thaliana* plants using *ACT2* as a constitutively expressed control gene. WT: *A. thaliana* Col-0; +A: pCambia2300::*FNR:ALDC*; +B: pCambia1300::*FNR:BDH1*; A1-9: Lines from T₂ generation transgenic plants transformed with pCambia2300::*FNR:ALDC*; B1-9: Lines from T₂ generation transgenic plants transformed with pCambia1300::*FNR:BDH1*. The dark circle in B indicates a very faint band.

The T₃ seed from all the positively expressing T₂ lines as well as all the double transformed T₂ lines were germinated and again tested for transgene incorporation and expression. Three lines of each of the single transformed T₃ lines (A3, A5, A9, B2, B4, B9) and three lines of the double transformed T₃ lines (AB2, AB4, AB6) indicated incorporation of the transgenes (Fig 3.8) after gDNA PCR, however, sqRT-PCT for expression analysis indicated that the majority of the T₃ lines showed either complete silencing or extremely low levels of transgene expression. Only lines A3 and B2 showed transgene expression under standard sqRT-PCR conditions, expression in line A9 was detected after 40 rounds of PCR, whilst lines A5, B9 and B4 still showed no amplification even after 40 cycles. For the double transgenics, line AB2 showed complete transgene silencing, whilst

expression could be detected for only the *ALDC* gene in line AB4 and the *BDH1* gene in line AB6 after 40 PCR cycles (Fig 3.9).

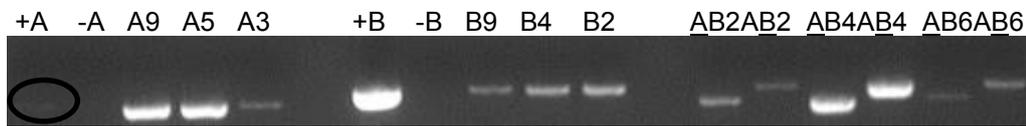


Figure 3.8: PCR of T₃ transgenic *A. thaliana* lines successfully transformed with the α -acetolactate decarboxylase (*ALDC*) gene from *A. niger* and/or the acetoin reductase/2,3-butanediol dehydrogenase (*BDH1*) gene from *S. cerevisiae*. -A;-B: *A. thaliana* Col-0; +A: pCambia2300::*FNR:ALDC*; +B: pCambia1300::*FNR:BDH1*; A3, A5, A9: Lines from T₃ generation transgenic plants transformed with pCambia2300::*FNR:ALDC*; B2, B4, B9: Lines from T₃ generation transgenic plants transformed with pCambia1300::*FNR:BDH1*; AB2, AB4, AB6: Lines from T₃ generation transgenic plants transformed with pCambia2300::*FNR:ALDC* and pCambia1300::*FNR:BDH1* respectively. The dark circle indicates a very faint band.

A possible explanation for the observed silencing could be that the requirement for α -acetolactate from the α -acetolactate decarboxylase was too much in the *ALDC* lines, resulting in gene silencing by the plant in an effort to survive. However, this does not provide an explanation for silencing observed in the *BDH1* lines, as these require acetoin and not α -acetolactate as a precursor for gene function. Meyer et al. (1992) reported that environmental factors such as high light intensity and temperature could induce transgene silencing, but in this study where plants were grown in a regulated growth room, such factors are unlikely to have been the cause of transgene silencing.

Transgene silencing has previously been observed in up to 50% of transgenic lines of *Arabidopsis*, petunia, rice and tobacco (Matzke et al., 1989; Kilby et al., 1992; Neuhuber et al., 1994; Van Blokland et al., 1994). All these studies used different genes and vectors, which suggest that transgene silencing is not due to a specific species, vector or gene (Meza et al., 2001). The transgenic lines which were silenced could definitely be effected by gene copy number and integration position in the chromosome (Linn et al., 1990; Hobbs et al., 1993) as well as the methylation pattern in that chromosome region of integration (Pröls and Meyer, 1992). The transgene sequence itself could also have an effect on silencing based on its GC content and possible methylation sites which makes it more or less compatible with the genomic organization of the host organism (Elomaa et al., 1995).

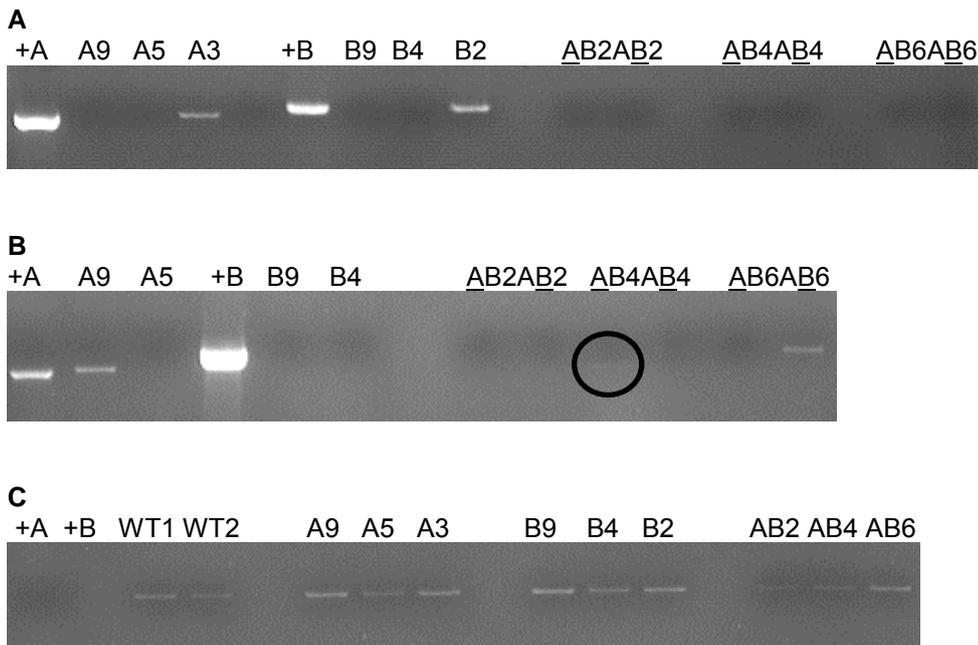


Figure 3.9: Steady state mRNA levels measured by semi-quantitative RT-PCR for **A)** 25 cycles and **B)** 40 cycles, of the α -acetolactate decarboxylase (*ALDC*) gene and/or acetoin reductase/2,3-butanediol dehydrogenase (*BDH1*) gene in T_3 transgenic *A. thaliana* plants using **C)** *ACT2* (25 cycles) as a constitutively-expressed control gene. WT: *A. thaliana* Col-0; +A: pCambia2300::*FNR:ALDC*; +B: pCambia1300::*FNR:BDH1*; A3, A5, A9: Lines from T_3 generation transgenic plants transformed with pCambia2300::*FNR:ALDC*; B2, B4, B9: Lines from T_3 generation transgenic plants transformed with pCambia1300::*FNR:BDH1*; AB2, AB4, AB6: Lines from T_3 generation transgenic plants transformed with pCambia2300::*FNR:ALDC* and pCambia1300::*FNR:BDH1*. The dark circle in B indicates a very faint band.

Downstream experiments that would have used low, medium and high expressing lines were modified to use only lines that showed transgene expression. Thus, for further studies, single lines A3, A6, B2 and B6 (all relatively high expressers in the T_2 generation) were used following confirmation of transgene expression in the T_3 generation (Fig 3.10).

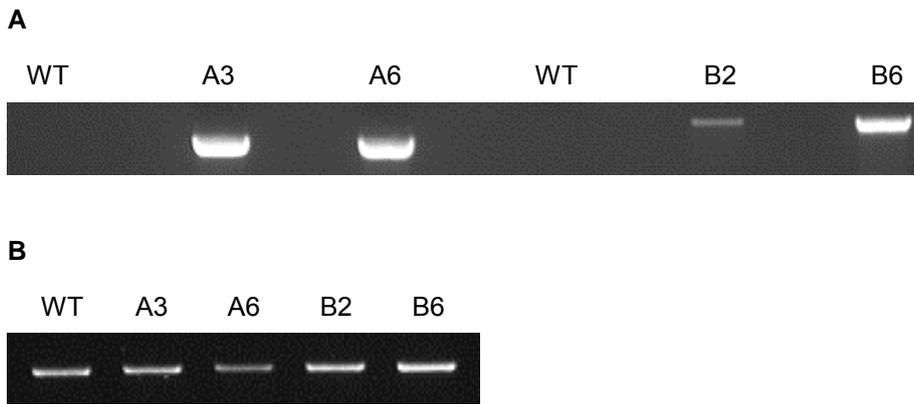


Figure 3.10: Semi-quantitative RT-PCR **A)** showing transcription of the α -acetolactate decarboxylase (*ALDC*) gene and acetoin reductase/2,3-butanediol dehydrogenase (*BDH1*) gene in T_3 transgenic *A. thaliana* plants using **B)** *ACT2* as a constitutively-expressed control gene. WT: *A. thaliana* Col-0; A3, A6: Lines from T_3 generation transgenic plants transformed with pCambia2300::*FNR:ALDC*; B2, B6: Lines from T_3 generation transgenic plants transformed with pCambia1300::*FNR:BDH1*.

Due to the silencing of genes in the T_3 double transgenic lines and not having any T_2 seed left for making new T_3 plants, new double transgenic lines had to be selected from T_1 putatively double transformed seed. Of the putatively double transformed T_1 seed remaining, only three seedlings survived the double antibiotic selection. Testing for gene insertion and expression indicated that line AB7 was a false positive as it only had insertion of the *BDH1* gene, however, lines AB8 and AB9 both had the *ALDC* and *BDH1* genes inserted (Fig 3.11 A). Lines AB8 and AB9 also expressed both genes (Fig 3.11 B) and were compared against *ACT2* as a constitutively-expressed control gene (Fig 3.11 C). Thus, lines AB8 and AB9 were seeded, and line AB7 was omitted from further studies. Due to time constraints and the possibility of gene silencing in the T_3 generation, the T_2 seed of the new double transformants were used for further experiments.

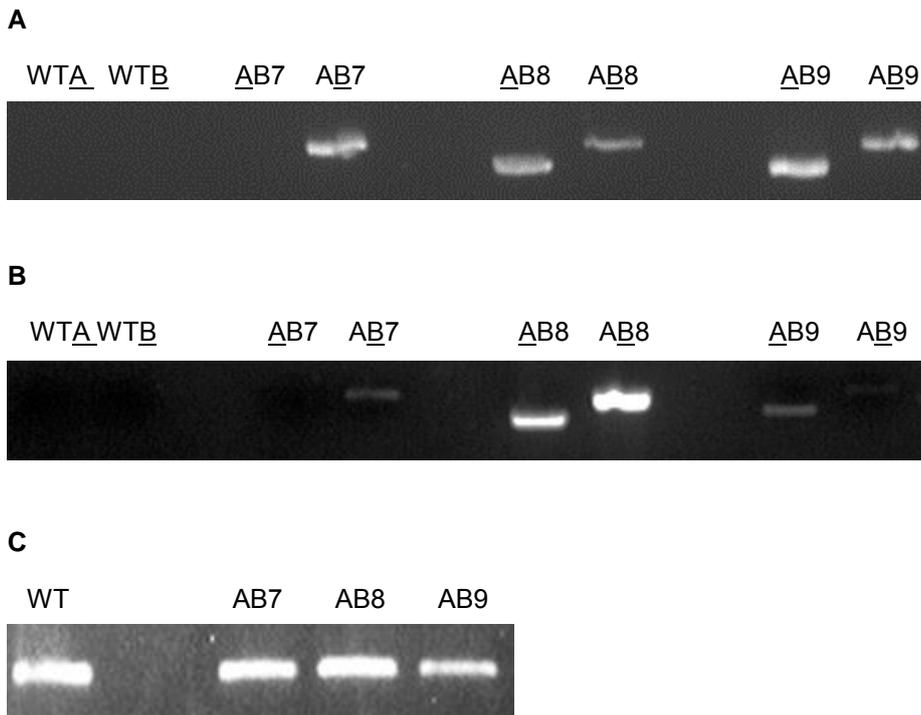


Figure 3.11: **A)** PCR of the α -acetolactate decarboxylase (*ALDC*) gene and acetoin reductase/2,3-butanediol dehydrogenase (*BDH1*) gene in T₁ transgenic *A. thaliana* plants using gDNA; sqRT-PCR amplification of transcripts of the **B)** α -acetolactate decarboxylase (*ALDC*) gene and acetoin reductase/2,3-butanediol dehydrogenase (*BDH1*) gene in T₁ transgenic *A. thaliana* plants and; **C)** *ACT2* as a constitutively-expressed control gene. WT: *A. thaliana* Col-0; AB7, AB8, AB9 represent T₁ generation transgenic plants transformed with pCambia2300::*FNR:ALDC* and pCambia1300::*FNR:BDH1*.

3.3 Plant growth experiments

3.3.1 Effects of synthetic acetoin and 2,3-butanediol on *Arabidopsis* plant growth *in vitro*

The growth stimulatory effect for exogenous application of 2,3-butanediol has been previously reported (Ryu et al., 2003). To test for synthetic acetoin and 2,3-butanediol function on *A. thaliana* in our growth conditions, we adapted the experiment. Plates with a center partition (I-plates) were changed to deeper standard tissue culture dishes, with the synthetic substance (mixed with lanolin wax, for decreased volatile release) being applied on a glass cover slip positioned in the center on top of the growth media (Fig 3.12). The growth time was also reduced to 10 days after exogenous application of the synthetic substances to allow the seedlings to grow without touching the cover lid as water accumulation on the leaves affected plant growth. Sealing the plates with parafilm led to seedling hyperhydricity, which was circumvented by using surgical tape. Lastly, day and night lengths were adjusted to fit our growth facilities.

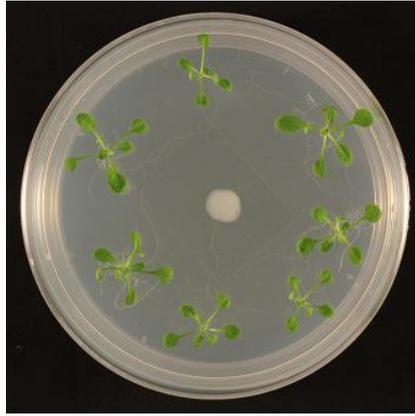


Figure 3.12: Exogenous synthetic compound plate set up.

Certain dilutions of exogenously applied synthetic 2,3-butanediol have been previously reported to enhance total leaf surface area of *A. thaliana* (Ryu et al., 2003). Since acetoin was also part of the major volatiles emissions from *B. subtilis* GB03 (Ryu et al., 2003), in this study a dilution series (20 μg , 200 ng, 2 ng, 20 pg and solvent alone) of both 2,3-butanediol and acetoin were set up independently and tested for growth-promoting effects under short day length (10h:14h, light:dark photoperiod) and long day length (16h:8h, light:dark photoperiod) conditions.

Under both growth conditions tested, both the synthetic acetoin and 2,3-butanediol exhibited different growth effects on the plants. Acetoin caused no growth promotion under the shorter day lengths (Fig 3.13 A) but significant increases with 20 μg , 200 ng and 2 ng under longer day length conditions (Fig 3.14 A). Under shorter day length conditions, 2,3-butanediol caused significant growth reduction (Fig 3.13 B) but no changes for the different concentrations under long day length conditions were observed (Fig 3.14 B). It should be noted that the acetoin (under long day conditions) and the 2,3-butanediol (under shorter day length conditions) generated somewhat inconsistent results between independently repeated experimental trails (data not shown). Despite numerous experiments to optimize different parameters, it proved impossible to consistently achieve completely identical results between independent experiments.

Under short day conditions, *Arabidopsis* flowering is delayed and leaf number increases (Cookson et al., 2007). Some genes which are sensitive to day length conditions also play a role in leaf development (Wang et al., 2003). It is thus possible that the two synthetic compounds interact with dissimilar genes at different developmental stages of the leaves, resulting in diverse growth effects under changing day lengths.

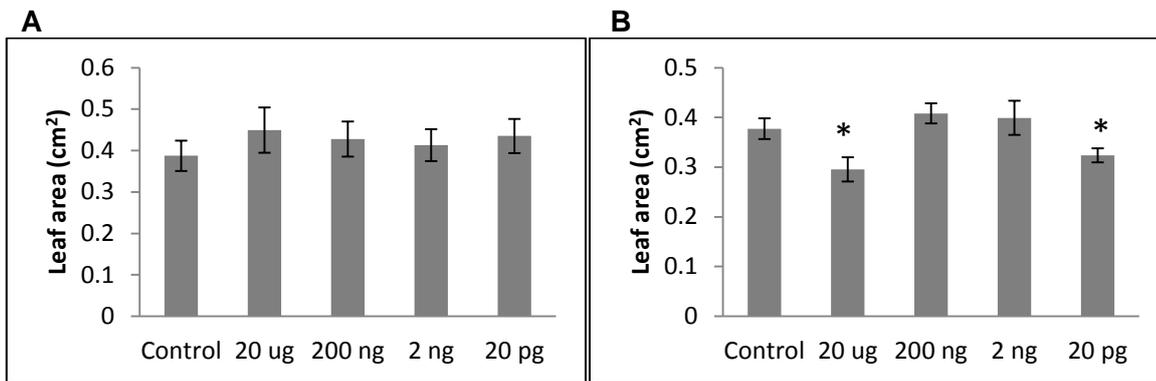


Figure 3.13: Total leaf surface area of 12 day old *Arabidopsis thaliana* Col-0 seedlings exposed for 10 days to synthetic **A)** acetoin; **B)** 2,3-butanediol grown under a 10h:14h light:dark photoperiod *in vitro*. Values represent the mean \pm SE (n = 5, each of the 5 plates had 5 seedlings, area measurement was taken as an average per plate) from one independent experimental trail. An asterisk indicates a value that was determined by t-test to be significantly different ($p \leq 0.05$) from the solvent control.

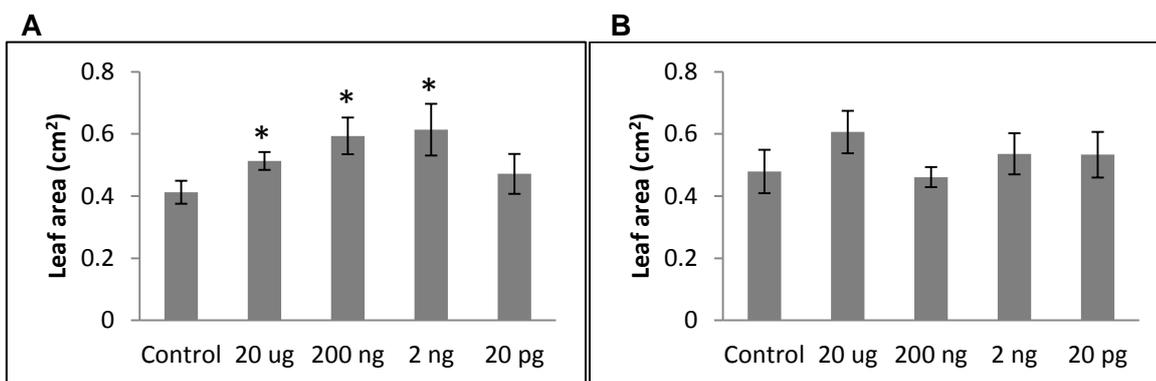


Figure 3.14: Total leaf surface area of 12 day old *Arabidopsis thaliana* Col-0 seedlings exposed for 10 days to synthetic **A)** acetoin; **B)** 2,3-butanediol grown under a 16h:8h light:dark photoperiod *in vitro*. Values represent the mean \pm SE (n = 5, each of the 5 plates had 5 seedlings, area measurement was taken as an average per plate) from one independent experimental trail. An asterisk indicates a value that was determined by t-test to be significantly different ($p \leq 0.05$) from the solvent control.

3.3.2 Growth of transgenic plants *in vitro*

3.3.2.1 Growth on non-selective media

While the growth stimulatory effect of *Bacillus amyloliquefaciens* IN937a and *Bacillus subtilis* GB03 volatile organic compounds has been previously reported (Ryu et al., 2003), no study to date has attempted producing the active volatiles (acetoin and 2,3-butanediol) *in planta*. After successful transformation of the *ALDC* and *BDH1* genes, responsible for the formation of acetoin and 2,3-butanediol respectively, into *A. thaliana*, growth comparison studies were executed between transgenic and untransformed control *A. thaliana* plants.

As a preliminary experiment, the transgenic T₃ lines A3, B2 and T₂ lines AB8, AB9 were tested against wild type *A. thaliana* Col-0 on tissue culture media without any antibiotic selection and grown in tissue culture conditions as previously described. Apart from total leaf surface area increasing (Fig 3.15 C; Fig 3.16 C), the fresh masses (Fig 3.15 A; Fig 3.16 A) and dry masses (Fig 3.15 B; Fig 3.16 B) of lines A3 and AB8 were significantly higher than those of the untransformed control plants, whilst the B2 line showed no changes from the wild type in any of the parameters tested. Dry mass measurements of single plants proved too small to be measured accurately, consequently five plants per plate were pooled for a measurement and calculated as an average per plant.

These results from 3 independent trails suggest that the A3 single transgenic line containing the *ALDC* gene grew bigger than the control plants (Fig 3.15) on account of the effect of this gene. The B2 line, however, although containing the *BDH1* gene, would not have been able to synthesise either acetoin, since it lacked the *ALDC* gene, or the 2,3-butanediol, since acetoin is required for the synthesis of 2,3-butanediol, and therefore showed similar levels of growth to the wild type plants (Fig 3.15).

Mixed results were obtained from the double transgenic lines expressing both *ALDC* and *BDH1*. Line AB8 grew significantly larger than control plants (Fig 3.15), as would be expected if these plants were able to produce both acetoin and 2,3-butanediol from *ALDC* and *BDH1* respectively. However, no significant differences in any of the growth parameters were observed for line AB9 (Fig 3.16). The levels of transgene expression in this line were considerably lower than in line AB8 (Fig 3.11), suggesting the possibility that the genes may have been expressed at insufficient levels to have an effect in this line. Due to time constraints it was only possible to carry out one experimental trail on the newly generated T₂ double transgenic lines.

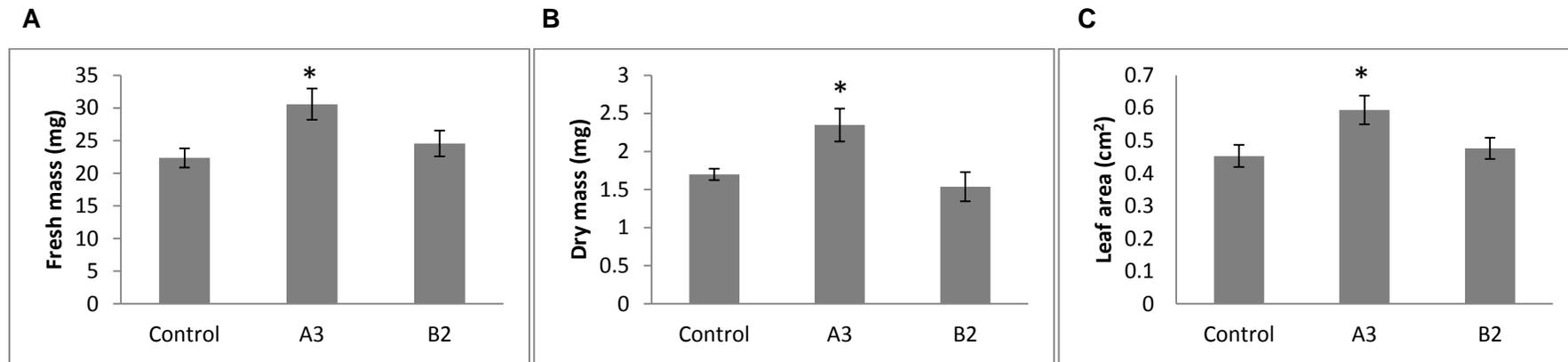


Figure 3.15: Growth of *Arabidopsis thaliana* plantlets *in vitro*. **A)** Whole plant fresh mass, **B)** Whole plant dry mass, and **C)** Leaf area of 14 day old tissue culture grown plantlets. Values represent the mean \pm SE A) n = 50; B) n = 10 (average dry mass of 5 plants per plate); C) n = 10 (average area of 5 plants per plate) from three independent experimental trails. An asterisk indicates a value that was determined by t-test to be significantly different ($p \leq 0.05$) from the control. Control: *A. thaliana* Col-0; A3: Line A3 from T₃ generation transgenic plants transformed with pCambia2300::*FNR:ALDC*; B2: Line B2 from T₃ generation transgenic plants transformed with pCambia1300::*FNR:BDH1*.

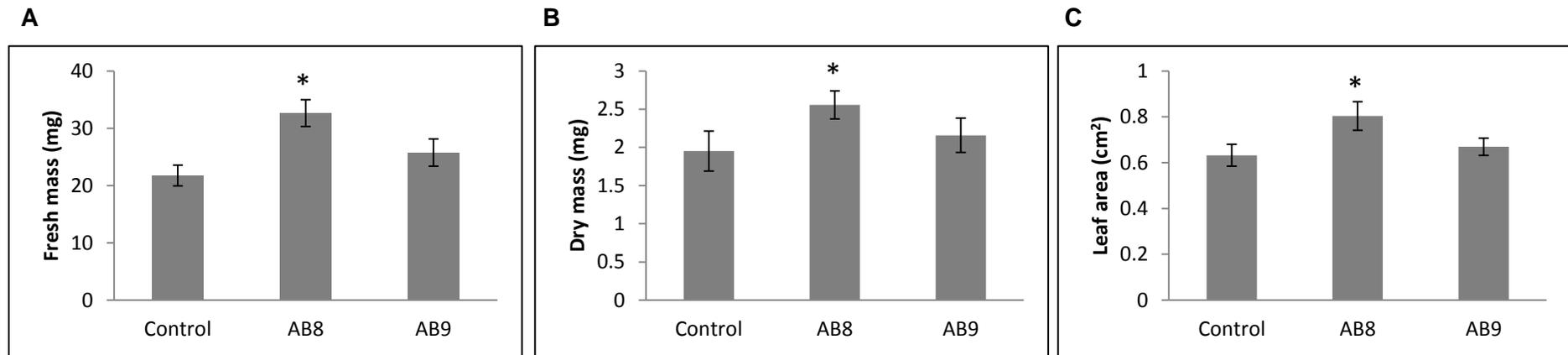


Figure 3.16: Growth of *A. thaliana* plantlets *in vitro*. **A)** Whole plant fresh mass, **B)** Whole plant dry mass, and **C)** Leaf area of 14 day old tissue culture grown plantlets. Values represent the mean \pm SE A) n = 25; B) n = 5 (average dry mass of 5 plants per plate); C) n = 5 (average area of 5 plants per plate) from one independent experimental trail. An asterisk indicates a value that was determined by t-test to be significantly different ($p \leq 0.05$) from the control. Control: *A. thaliana* Col-0; AB8, AB9: Lines from T₂ generation transgenic plants transformed with pCambia2300::*FNR:ALDC* and pCambia1300::*FNR:BDH1*.

3.3.2.2 Growth on selective media

Due to the possibility of a heterozygous seed population still being present in the T₃ generation, another preliminary experiment was conducted to determine whether the transgenic seedlings grew at the same rate on antibiotic selection media as untransformed control *A. thaliana* Col-0 on normal tissue culture media, in order to determine if the growth experiments could be repeated on selective media. Results indicated that there was no clearly visible developmental difference within the first five days between untransformed control plants (Fig 3.17 A), the single transgenic plants (A3, A6, B2, B6) and the T₂ generation double transgenic, AB8 line (Fig 3.17 B-F).

However, the T₂ generation double transgenic, AB9 line showed distinct growth retardation on the double selection medium when compared with control plants on non-selective medium, with only the cotyledons being visible after 9 days (Fig 3.17 H), whereas the first true leaves emerged in WT individuals after 5 days (Fig 3.17 A). Due to time constraints, line AB9 was omitted from further studies. Other independently double transformed lines are being generated for future investigation.

To test for differences in growth between the untransformed control plants and the transgenic lines, five day old seedlings (Fig 3.17 A-F) which survived the above-mentioned selection were carefully transferred to normal tissue culture growth medium as previously described and grown until they were a total of 14 days old. Total leaf surface area, whole fresh mass and whole dry mass were then measured. Results obtained (data not shown) were inconsistent with previous results without selection pressure, indicating that the seedlings could have been physiologically stressed when transferred to fresh media, although extreme caution was taken when doing so.

When the above-mentioned transgenic lines were subjected to selection media for the full 14 days of growth, leaf area measurements indicated that the single lines exhibited the same growth trend (Fig 3.18) as those on non-selection media (Fig 3.15). Lines A3 and A6 had significantly greater leaf area compared to that of the control plants, whilst lines B2 and B6 behaved similarly to the control (Fig 3.18). Although the double antibiotic medium seemed to cause no visible changes in growth after 5 days, it appeared that this selection regime proved to be too intense over the full 14 day growth period as the growth of all AB8 plants was significantly retarded (Fig 3.18).

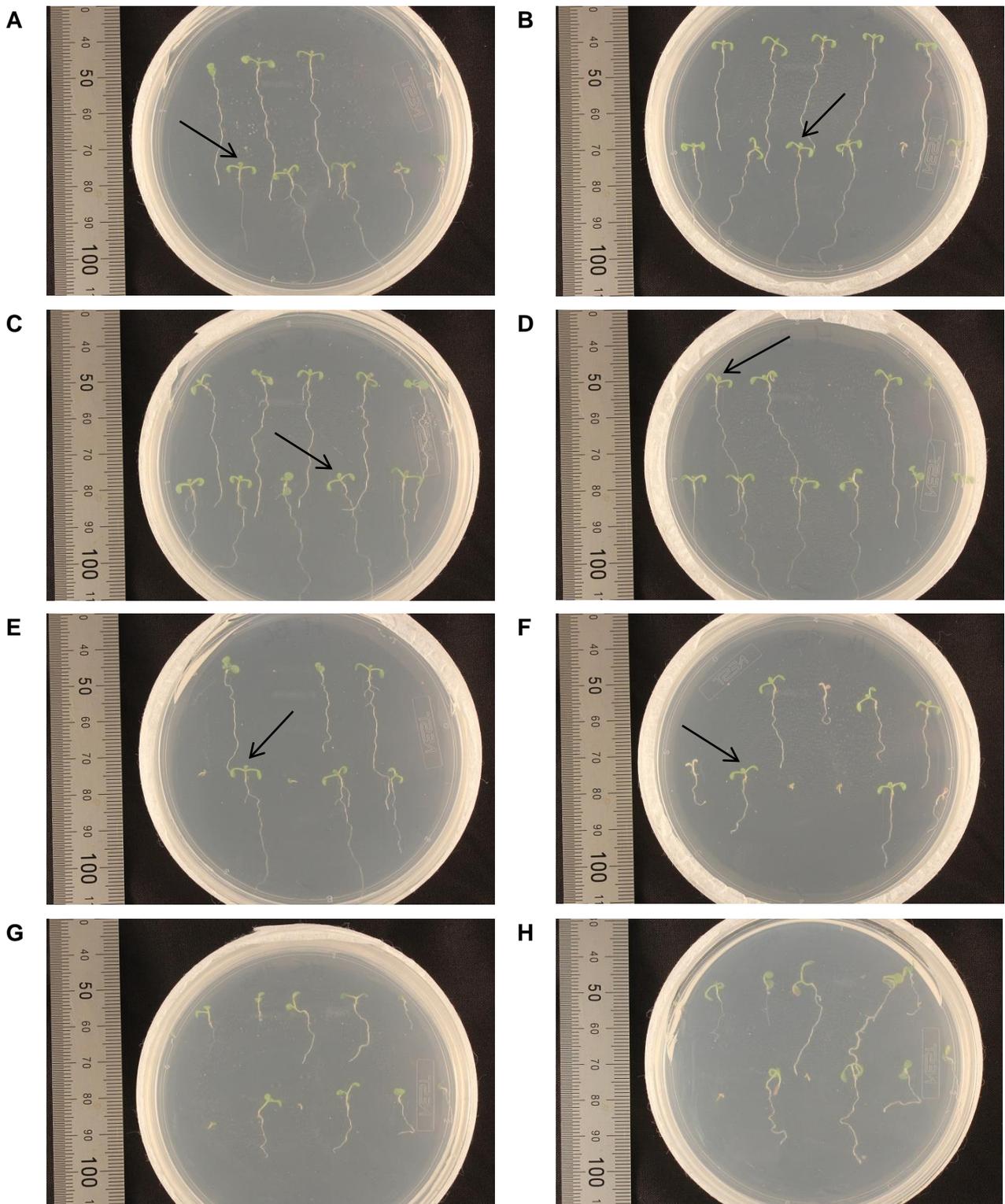


Figure 3.17: Growth of five day old *A. thaliana* plantlets *in vitro*. **A)** *A. thaliana* Col-0, no selection; **B)** line A3 and **C)** line A6 from T_3 generation transgenic plants transformed with pCambia2300::*FNR:ALDC* on kanamycin selection; **D)** line B2 and **E)** line B6 from T_3 generation transgenic plants transformed with pCambia1300::*FNR:BDH1* on hygromycin selection; **F)** line AB8 and **G)** line AB9 from T_2 generation transgenic plants transformed with pCambia2300::*FNR:ALDC* and pCambia1300::*FNR:BDH1* on kanamycin and hygromycin selection; **H)** Line AB9, 9 days old. Arrows indicate first true leaves.

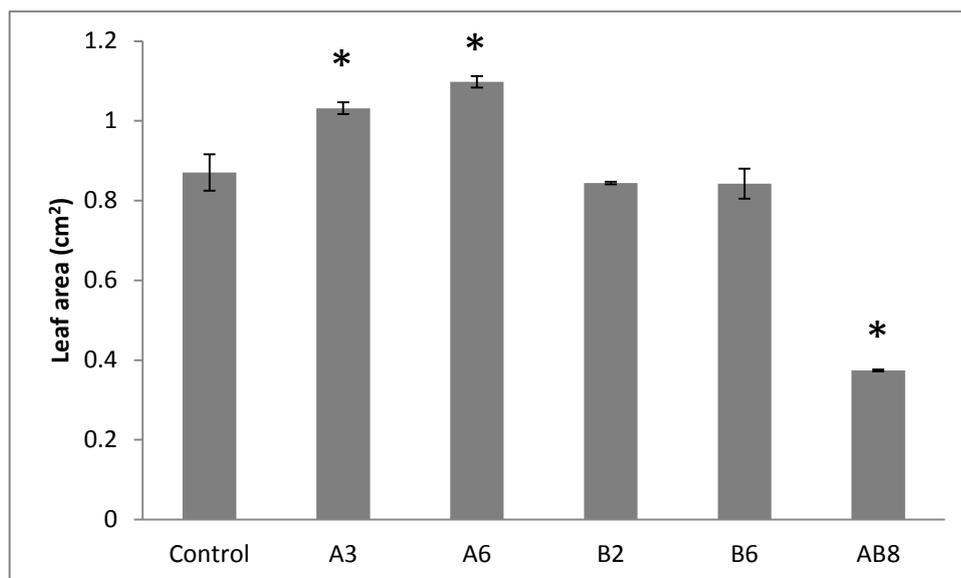


Figure 3.18: Leaf area of *Arabidopsis thaliana* plants grown *in vitro* for 14 days. Control wild type plants were grown on the standard medium, the transgenic lines were grown on kanamycin and/or hygromycin containing selective media. Values represent the mean \pm SE ($n = 3$, each plates had 10 seedlings, area measurement was taken as an average per plate) from one independent experimental trail. An asterisk indicates a value that was determined by t-test to be significantly different ($p < 0.05$) from the control. Control: *A. thaliana* Col-0; A3, A6: Lines from T₃ generation transgenic plants transformed with pCambia2300::*FNR:ALDC*; B2, B6: Lines from T₃ generation transgenic plants transformed with pCambia1300::*FNR:BDH1*; AB8: Line AB8 from T₂ generation transgenic plants transformed with pCambia2300::*FNR:ALDC* and pCambia1300::*FNR:BDH1*.

Growth experiments in general, including previously published synthetic compound experiments, were difficult to reproduce with identical results in our growth conditions. Despite identical experimental set up between repeats, control plants growth data varied slightly between experiments, by growing to different sizes. Thus, control samples needs to be more consistent before different experiments could be truly compared to one another. Although some inconsistencies were observed between different experimental repeats, a clear trend was observed for the single transgenic lines when compared against the untransformed control plants. The transgenic *ALDC* lines grew bigger than the control plants, suggesting that the presence of the α -acetolactate decarboxylase transgene allowed these plants to convert acetolactate to acetoin, which then positively affected their growth. The *BDH1* lines behaved the same as untransformed control plants, since the absence of acetoin in these lines meant that no 2,3-butanediol could be generated.

To improve experiments like these in the future, they should be set up in controlled growth chambers with higher quality lights and more closely regulated day and night temperatures, rather than in a walk-in tissue culture growth room. Caution should also be taken to add exactly the same volume of growth media between different tissue culture dishes. Arrangement of the plates could also be completely randomized in the growth chamber, to eliminate any possibilities of light/temperature gradients being present.

3.4 Analysis of transgenic plants

3.4.1 Branched-chain amino acids analysis

The ability to synthesize BCAA *de novo* is unique to bacteria, archaea, fungi and plants (Binder, 2010), where a common pathway exists for the synthesis of α -acetolactate which is an intermediate in the production of the BCAA, i.e. leucine and valine. Alpha-acetolactate is formed from two molecules of pyruvate by acetohydroxyacid synthase (AHAS), also known as acetolactate synthase (ALS, EC 2.2.1.6) (Umbarger, 1978; Binder, 2010). When ALS/AHAS is disturbed in plants by herbicide chemicals (ALS-inhibitors), which include sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs) pyrimidinyl oxybenzoates (POBs), and sulfonylamino carbonyl triazolinones (SCTs) (Shaner, 1999), a reduced level of acetolactate is produced which in turn decreases the synthesis of leucine, valine and isoleucine, which will cause the gradual death of the whole plant due to the lack of proper DNA synthesis (Tranel and Wright, 2002).

A slight concern for this study was that due to the α -acetolactate decarboxylase being constitutively expressed under the CaMV35S promoter, a possible decrease in BCAA levels could arise due to overexpression of α -acetolactate decarboxylase in *A. thaliana*, or any other plant for that matter, by using the same precursor for acetoin production as for the start of leucine and valine production i.e. acetolactate. Fortunately, in this study none of the transgenic lines grew less than the untransformed control plants (Fig 3.18), except under prolonged selection on double antibiotic media, suggesting that the BCAA levels were still suitable for growth. When repeating a similar type of study, care should be taken with the strength of the promoter being used to overexpress α -acetolactate decarboxylase. A very strong promoter could possibly have more detrimental than positive effects by causing depletion of the acetolactate levels for the production of acetoin, leading to hampered growth or even death by indirectly acting as an internal herbicide.

Free amino acid levels were extracted and tested from Jiffy-7[®]-grown leaf material of transgenic lines. Pot-grown plants were utilized rather than the tissue-cultured plants used in the growth experiments due to the amounts of tissue required for the amino acid measurements. No differences in either leucine or valine were found in the transgenic lines compared with the control lines. However, isoleucine levels in the B2 line were significantly reduced (Fig 3.19). Since the data

was obtained using only two replicates, this finding should be confirmed using a greater number of replicates, however, the data does not appear to suggest that there is cause for concern. A partially-deficient isoleucine mutant in *A. thaliana* displayed a much shorter root length compared to control seedlings (Yu et al., 2013), this was not observed in this study where the root lengths of lines B2 and B6 (Fig 3.17 D,E) were comparable to those of untransformed control *A. thaliana* seedlings (Fig 3.17 A).

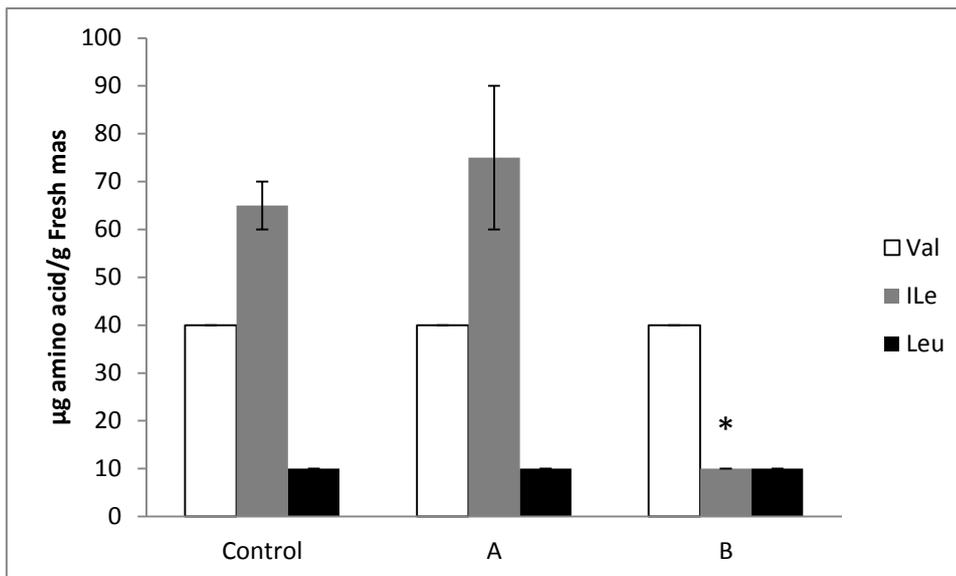


Figure 3.19: Amino acid content per gram fresh mass of transgenic *Arabidopsis* leaf material. Values represent the mean \pm SE ($n = 2$). An asterisk indicates a value that was determined by t-test to be significantly different ($p < 0.05$) from the control. Control: *A. thaliana* Col-0; A: Line A3 from T_3 generation transgenic plants transformed with pCambia2300::*FNR:ALDC*; B: Line B2 from T_3 generation transgenic plants transformed with pCambia1300::*FNR:BDH1*.

3.4.2 Detection of VOCs

3.4.2.1 Enzyme assays

3.4.2.1.1 Enzymatic assay of α -acetolactate decarboxylase

To date no studies have been conducted on *A. thaliana* to determine whether acetoin is present in wild type plants. Acetoin enzymatic assays have been carried out on cell cultures of carrot, maize, rice and tobacco (Forlani et al., 1999). Due to time limitations cell cultures were not made in this study, thus a different method which is based on the Voges-Proskauer test (Voges and Proskauer,

1898) was tested for the detection of acetoin in plants in this study. Crude protein was extracted from transgenic *ALDC* and untransformed control plants and tested for the presence of acetoin by a colometric method. No significant differences were observed in the optical density at 522 nm between the control sample and *ALDC* sample after addition of the naphthol/creatine colour reagent (Table 3.1).

Table 3.1: Optical density values at 522 nm of enzymatic assay of α -acetolactate decarboxylase after addition of colour reagents to prepared samples of untransformed control plants and line A3 from T₃ generation *ALDC* transgenic plants.

	Control	<i>ALDC</i>
OD 522 nm (n = 5)	0.2138	0.2116
SE	± 0.001393	± 0.001435

3.4.2.1.2 Enzymatic assay of acetoin reductase activity

To test for acetoin reductase activity ($\text{Acetoin} + \text{NADH} \rightarrow 2,3\text{-butanediol} + \text{NAD}^+$), crude protein was extracted from transgenic *BDH1* and untransformed control *A. thaliana* leaf material and assayed for acetoin reductase activity as described in Section 2.26. As expected, no decrease in absorbance at 340 nm was observed when crude protein (Fig 3.20 A) or NADH (Fig 3.20 B) was omitted from the assay buffer. When all components were present in the assay there was a decrease in NADH levels, suggesting that acetoin-dependent oxidation of NADH to NAD^+ had occurred (Fig 3.20 C). To confirm this finding, the assay buffer was tested without the addition of acetoin (Fig 3.20 D). The same rate of oxidation took place when all components were present in the assay buffer compared to when acetoin was omitted. Identical trends were also observed in the untransformed control samples (data not shown). These observations indicate that some other compound(s) in the crude protein extract also use(s) NADH. Although positive results were obtained by Nicholson (2008) using this method on bacterial cultures, the NADH-coupling approach to measure acetoin reductase activity in the plant crude extracts could not be utilized due to this alternate activity.

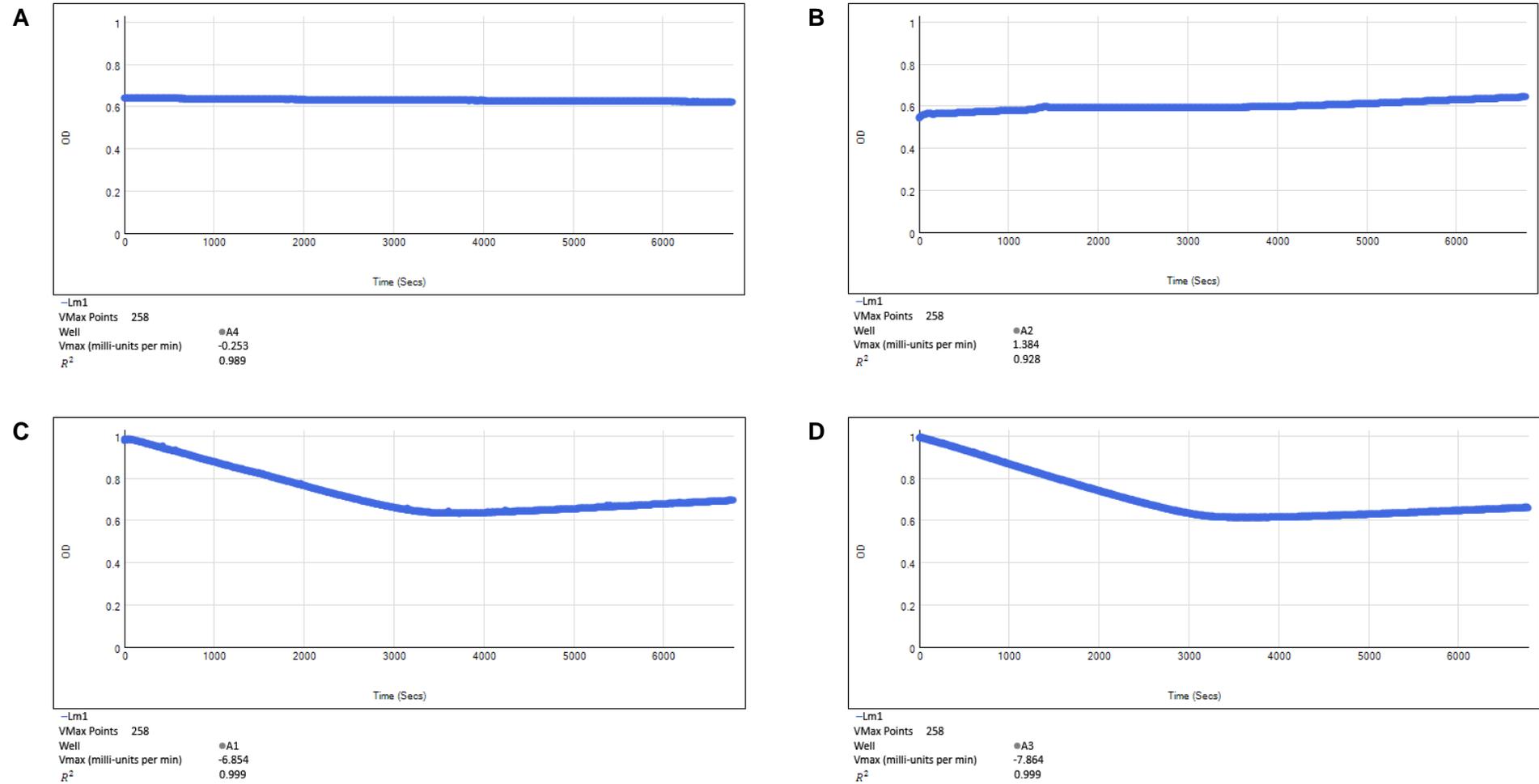


Figure 3.20: Acetoin reductase enzymatic assay of T₃ generation transgenic plants transformed with pCambia1300::*FNR:BDH1*, reduced nicotinamide adenine dinucleotide (NADH) oxidation measured at 340 nm. **A)** no protein added; **B)** no NADH added; **C)** all components added; **D)** no acetoin added.

3.4.2.2 Determining VOC released from transgenic lines using GC-MS

Volatile analysis in the headspace, the air above the sample in a chromatography vial, is a far better method for determining volatiles emitted by plants than solvent extractions (Tholl et al., 2006). To determine whether acetoin was released from the *ALDC* transgenic lines, static headspace GC-MS analysis was performed with a SEP (Section 2.28). When the acetoin standard was analyzed a peak was observed at a retention time of 4.93 min (Fig 3.21). Mass spectrum analysis identified the standard peak as acetoin (Fig 3.22). The total ion trace of the transgenic *ALDC Arabidopsis* leaf material is depicted in Fig 3.23. After enlarging the retention time period between 3 and 7 min (on the total ion trace chromatogram, Fig 3.23), no peak was observed at the expected 4.93 min mark, which gave clear results that no acetoin was present in the transgenic *ALDC Arabidopsis* headspace sample (Fig 3.24). No detectable amounts of acetoin were released from either whole leaves or ground-up leaves of the *ALDC* transgenic lines into the collection chamber in a 24 h period.

Due to time constraints, further optimization of the GC-MS detection method was not possible. Future methods for improvement include using more starting leaf material and heating the material to release semi-volatiles that might otherwise be trapped. An improved detection method could be conducted using dynamic head space analysis over a longer period of time, as described by Tholl et al. (2006). Volatiles could also be detected as described by Huang et al. (2011), where the closed-loop stripping method from Donath and Boland (1995) was used for the detection of volatiles from low emitting plants. Kusano et al. (2013) investigated the composition of VOCs under different growth conditions where they developed a headspace-solid-phase microextraction-gas chromatography-time-of-flight-mass spectrometry (HS-SPME-GC-TOF-MS) method, which could also be considered for future analysis. The transgenic *BDH1* lines and double transformed lines could also then be analysed for acetoin and 2,3-butanediol release.

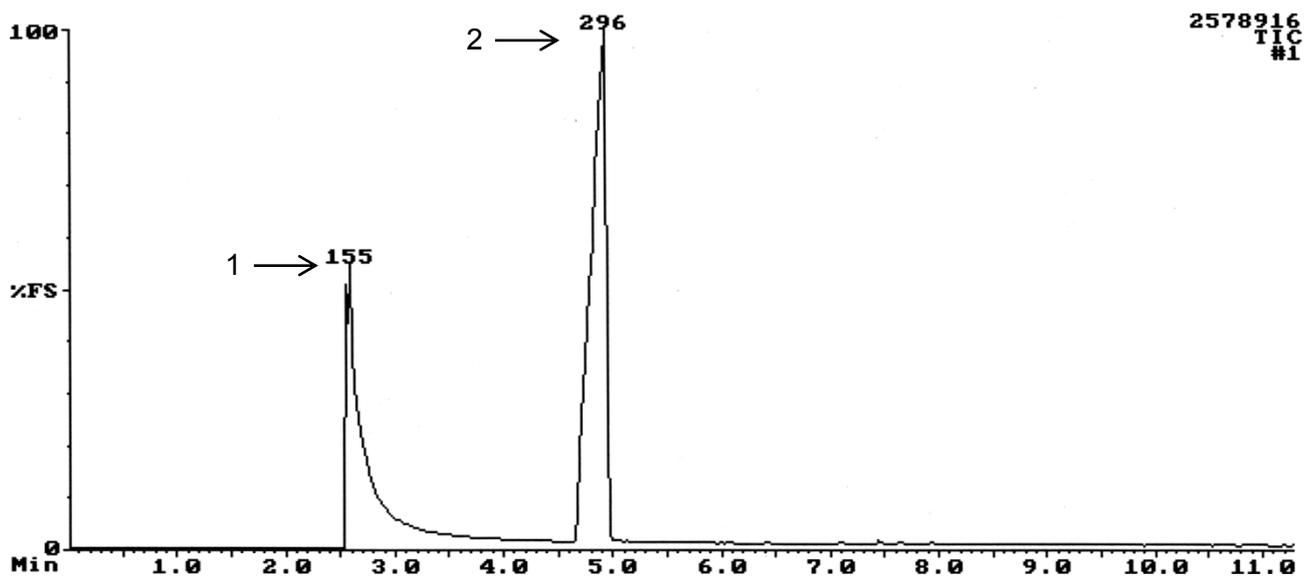


Figure 3.21: Total ion chromatogram (TIC) obtained by gas chromatography-mass spectrometry (GC-MS) analysis of a synthetic acetoin sample analyzed on a Zebron™ ZB-5MS column. 1) Peak of residual air remaining in the injector after introduction of the sample enrichment probe (SEP); 2) Scan number and retention time in s of the synthetic acetoin standard.

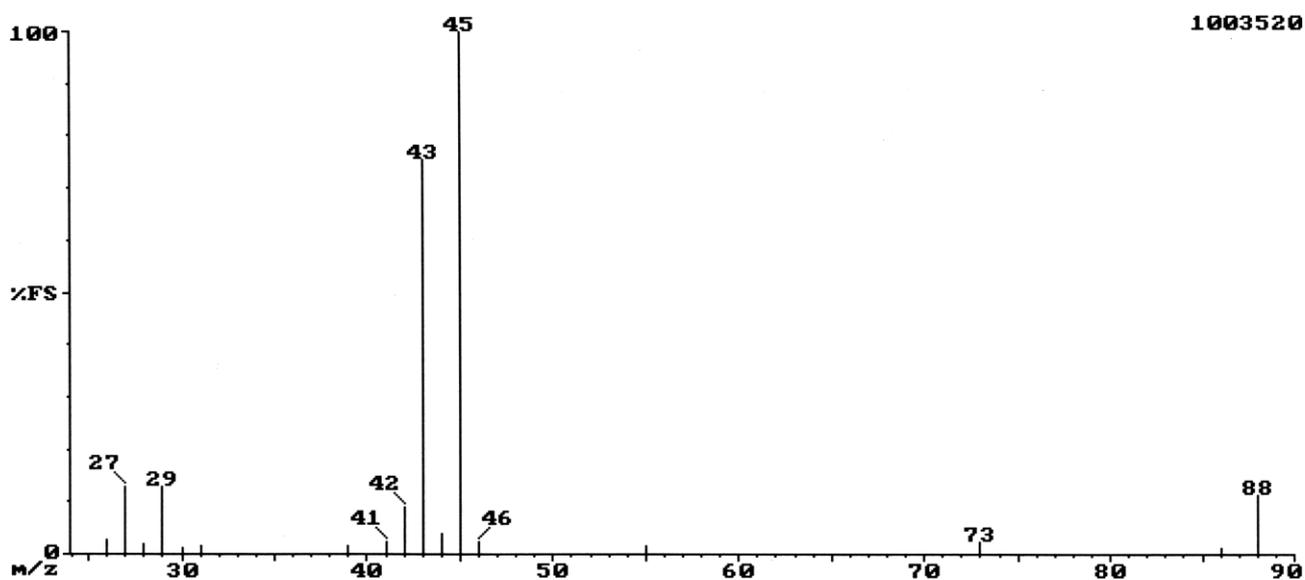


Figure 3.22: Mass spectrum of acetoin eluting at 4.93 min of the total ion chromatogram (TIC) depicted in Fig 3.21.

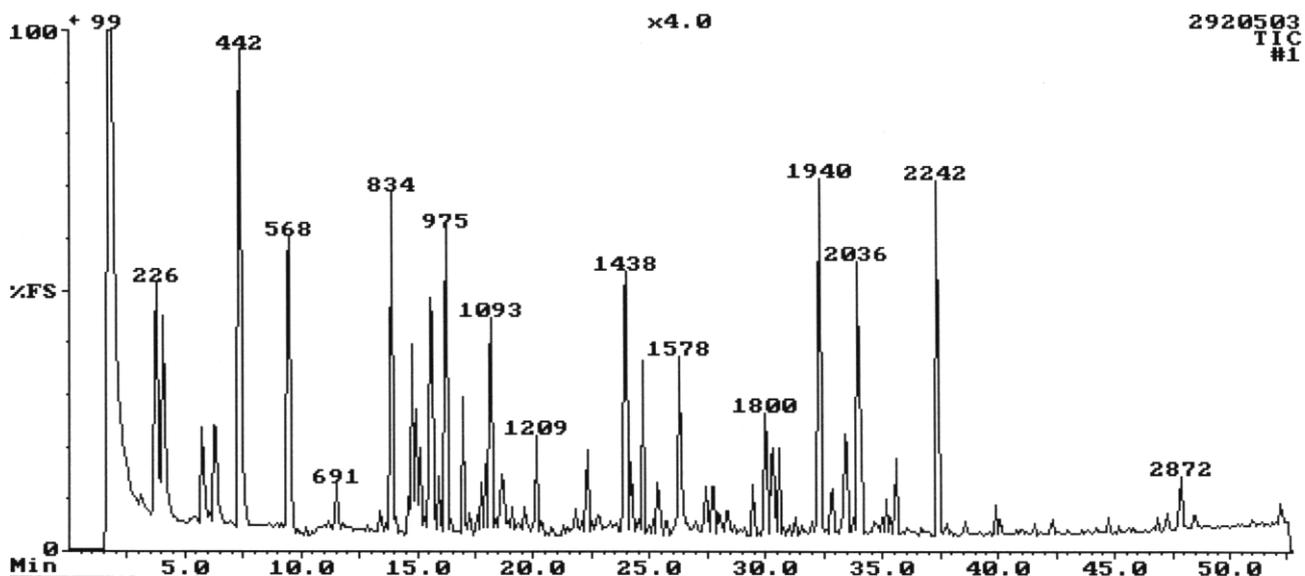


Figure 3.23: Total ion chromatogram (TIC) of the headspace volatiles from transgenic *ALDC Arabidopsis* leaf samples analyzed on a Zebron™ ZB-5MS column.

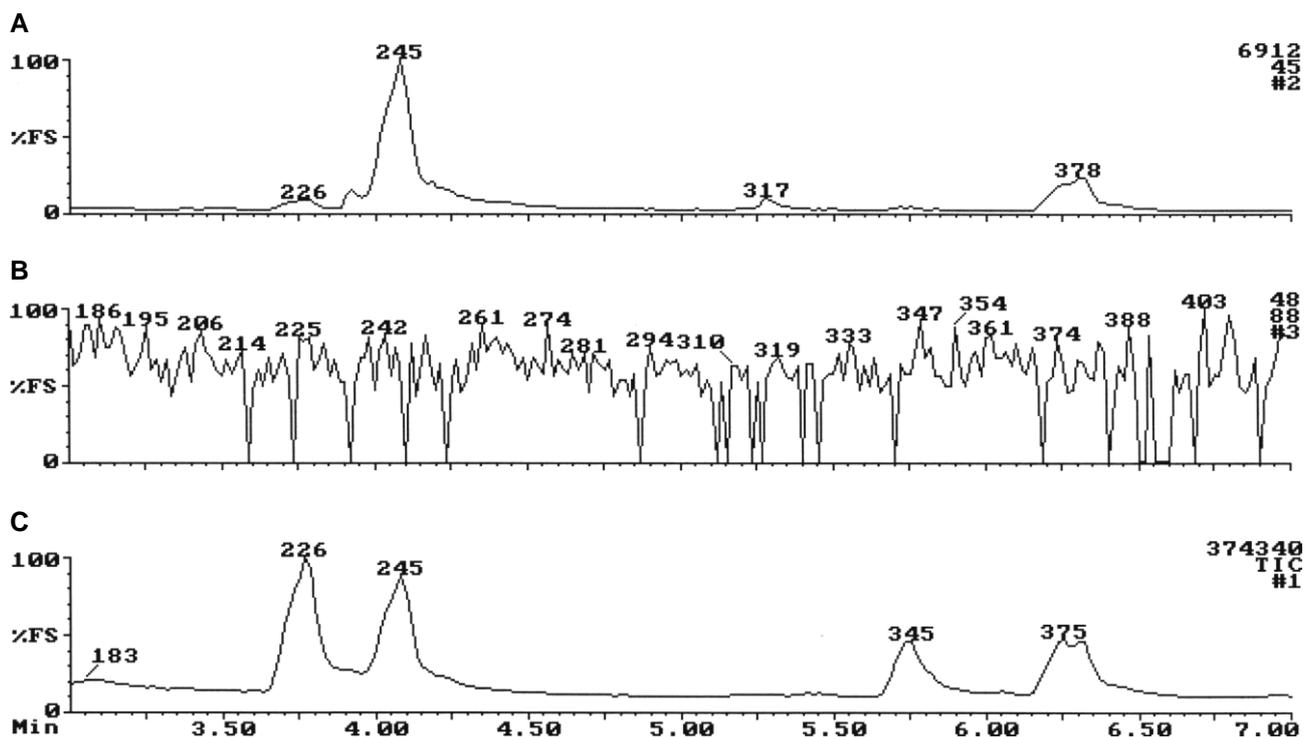


Figure 3.24: Chromatograms of the headspace volatiles from transgenic *ALDC Arabidopsis* leaf samples analyzed on a Zebron™ ZB-5MS column of retention times 3 to 7 min (enlarged from Fig 3.23). The analysis was independently repeated three times. **A)** Selected ion chromatogram of the base peak of acetoin; **B)** Selected ion chromatogram of the molecular ion ($m/z = 88$); **C)** Total ion chromatogram (TIC); (Peak expected at 4.93 min, as seen in Fig 3.21, for chromatogram A, B and C).

3.4.2.3 High-performance liquid chromatography (HPLC) detection of acetoin and 2,3-butanediol

In combination with the enzyme activity assays and headspace GC-MS methodologies used to detect acetoin and/or 2,3-butanediol, HPLC was also performed to detect the presence of acetoin in transformed *A. thaliana* plants. Transgenic leaf tissue samples were separated on an Aminex HPX-87H column using a simple isocratic HPLC method. Acetoin and 2,3-butanediol presence were tested for in the samples using a DAD at 278.8 nm and a RID, respectively, and identified by the elution times from the synthetic standards.

Comparing the HPLC results of the synthetic acetoin (Fig 3.25) standards against that of the untransformed control *A. thaliana* plants, it is clearly observable that no acetoin was present in the control sample (Fig 3.26) as no peaks at DAD 278 nm and RID, respectively, were detected at the corresponding elution times of the acetoin standard. Examination of the control sample spiked with synthetic acetoin (Fig 3.27) revealed a strong peak at DAD 278 nm (+21.4 min) compared to that of the control acetoin sample (Fig 3.25), which indicated that acetoin is still detectable and not influenced by the presence of other compounds in the extract. No corresponding peaks, at the expected retention time of 21 min, were observed between the acetoin control sample (Fig 3.25) and the transformed *ALDC* sample (Fig 3.28), suggesting that no acetoin is present in the transformed sample or that the HPLC system is not sufficiently sensitive to detect possibly minute amounts of the volatiles produced by the transgenic plants. Another alternative possibility is that the acetoin is immediately used after being produced and broken down to single elements i.e: C, H and O.

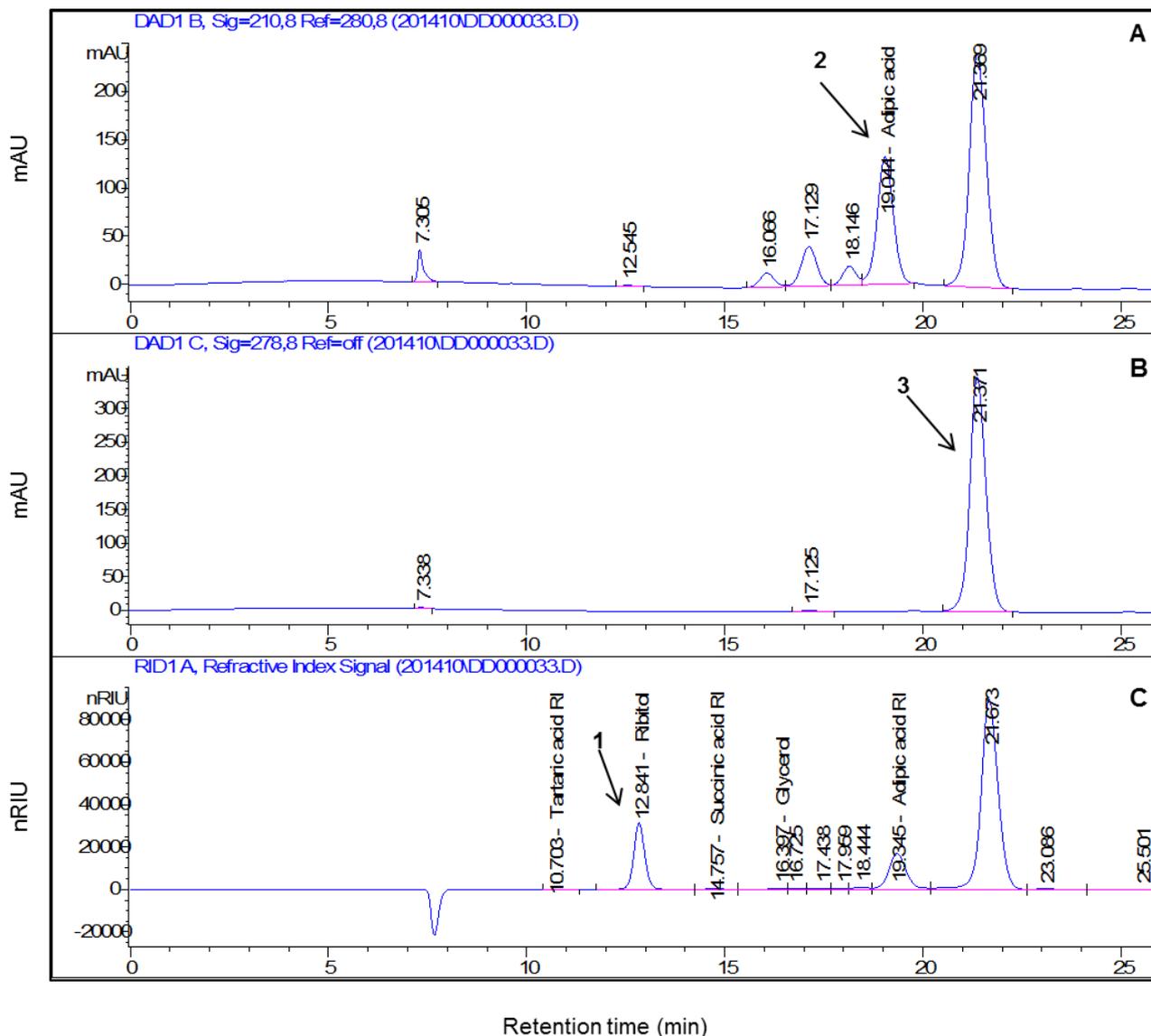


Figure 3.25: High-performance liquid chromatography (HPLC) chromatogram of an aqueous standard acetoin solution with adipic acid and ribitol as internal standards (IS) detected using a diode array detector (DAD) at **(A)** 210 nm, **(B)** 278.8 nm, and **(C)** a refractive index detector (RID). Ribitol [1] and Adipic acid [2] peaked at ± 12 min and ± 19 min, respectively. The retention time for the acetoin standard [3] was at ± 21 min. Units of X-axis is minutes, units of Y-axis is milli-absorbance units (mAU) detected by the DID at 210 and 278.8 nm or nano Refractive Index Units (nRIU) detected by the RID.

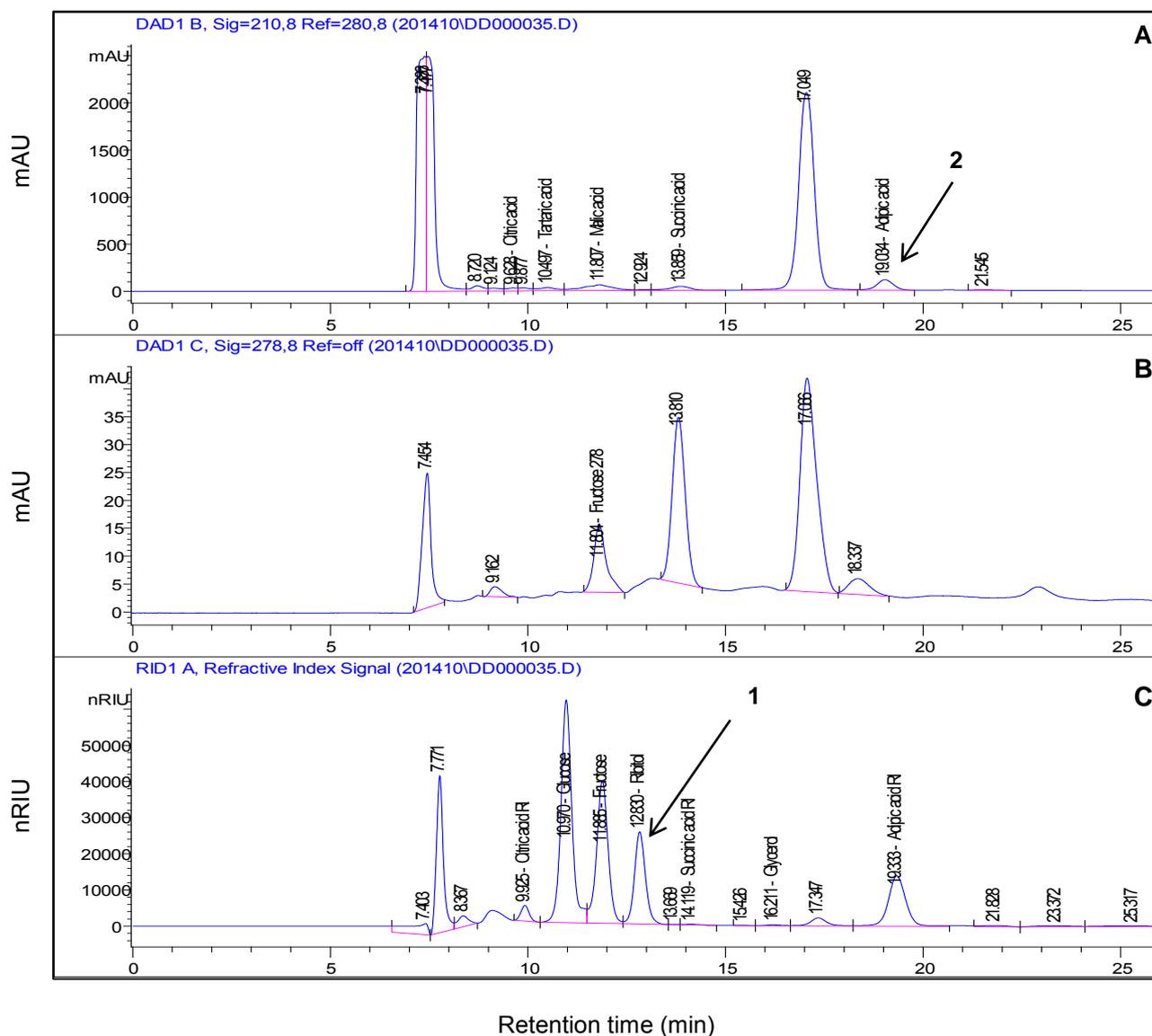


Figure 3.26: High-performance liquid chromatography (HPLC) chromatogram of an untransformed *Arabidopsis thaliana* leaf extract with adipic acid and ribitol as internal standards (IS) detected using a diode array detector (DAD) at **(A)** 210 nm, **(B)** 278.8 nm, and **(C)** a refractive index detector (RID). Ribitol [1] and Adipic acid [2] peaked at ± 12 min and ± 19 min, respectively. Units of X-axis is minutes, units of Y-axis is milli-absorbance units (mAU) detected by the DID at 210 and 278.8 nm or nano Refractive Index Units (nRIU) detected by the RID.

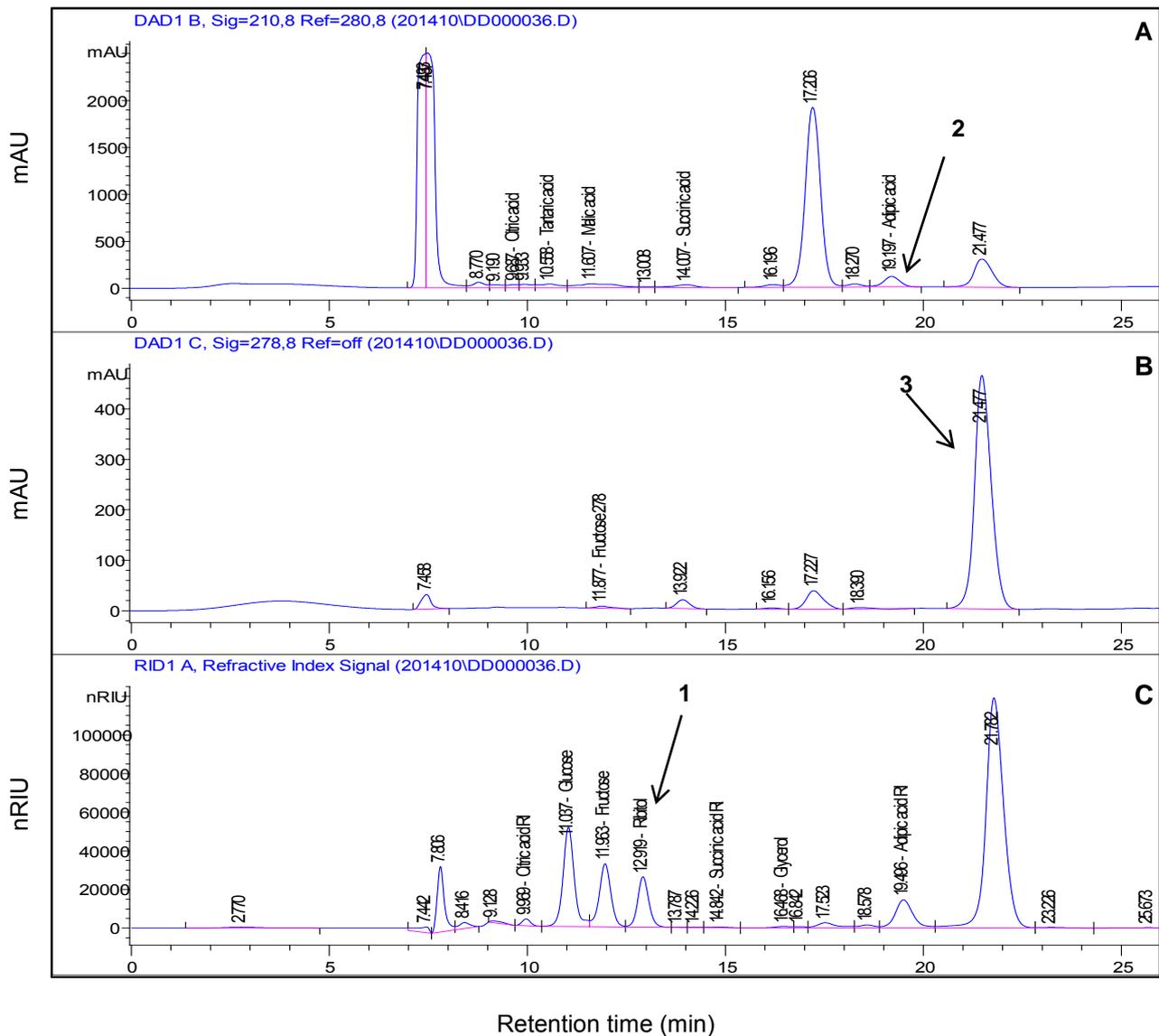


Figure 3.27: High-performance liquid chromatography (HPLC) chromatogram of an untransformed *Arabidopsis thaliana* leaf extract spiked with an acetoin standard with adipoic acid and ribitol as internal standards (IS) detected using a diode array detector (DAD) at **(A)** 210 nm, **(B)** 278.8 nm, and **(C)** a refractive index detector (RID). Ribitol [1] and Adipoic acid [2] peaked at ± 12 min and ± 19 min, respectively. The retention time for the acetoin standard [3] was at ± 21 min. Units of X-axis is minutes, units of Y-axis is milli-absorbance units (mAU) detected by the DID at 210 and 278.8 nm or nano Refractive Index Units (nRIU) detected by the RID.

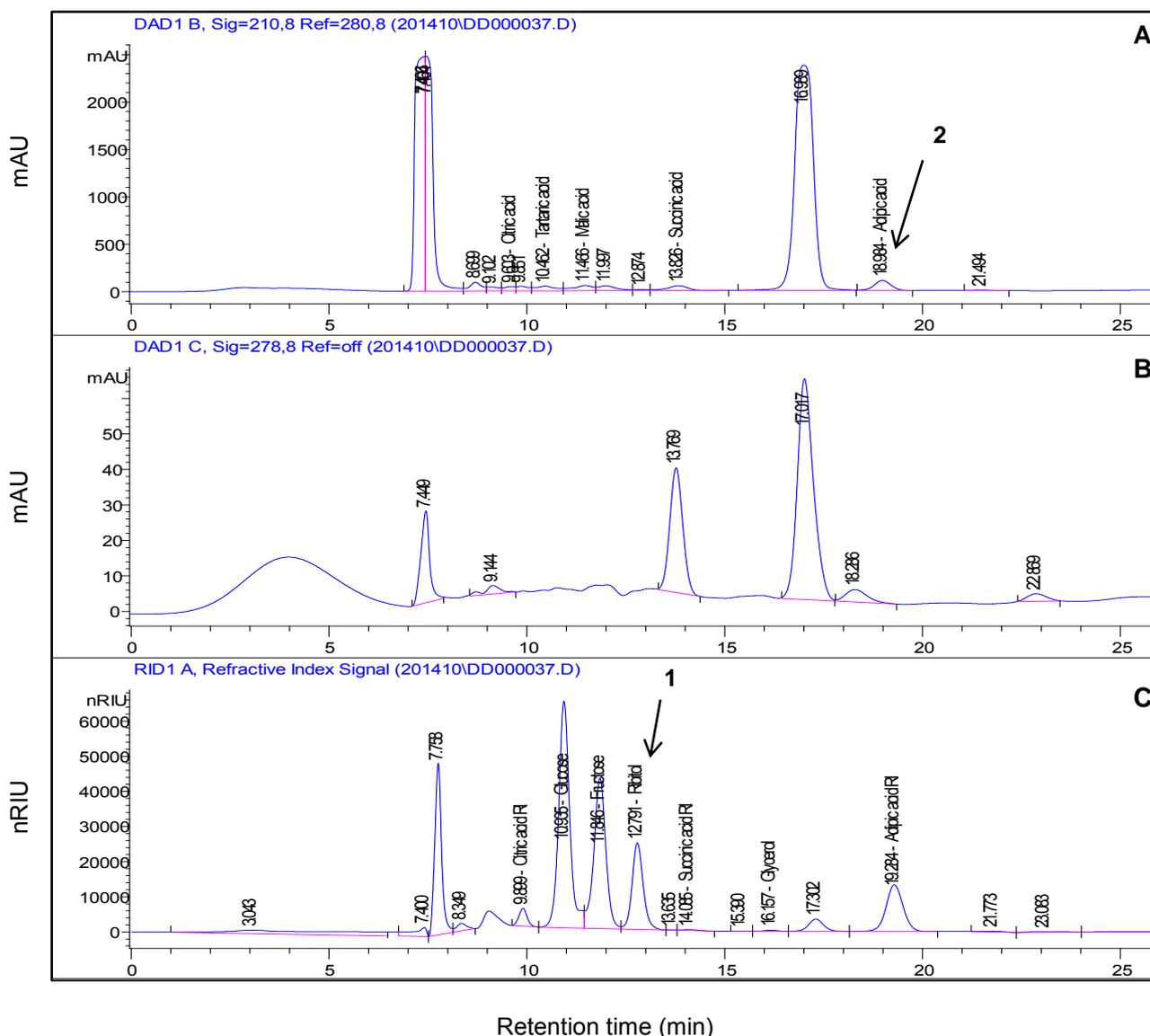


Figure 3.28: High-performance liquid chromatography (HPLC) chromatogram of an *ALDC* transgenic *Arabidopsis thaliana* leaf extract with adipic acid and ribitol as internal standards (IS) detected using a diode array detector (DAD) at **(A)** 210 nm, **(B)** 278.8 nm, and **(C)** a refractive index detector (RID). Ribitol [1] and Adipic acid [2] peaked at ± 12 min and ± 19 min, respectively. Units of X-axis is minutes, units of Y-axis is milli-absorbance units (mAU) detected by the DID at 210 and 278.8 nm or nano Refractive Index Units (nRIU) detected by the RID.

Several different systems were implemented in this study for the detection of VOCs, i.e. enzymatic assays, GC-MS and HPLC. The enzymatic assays were not a practical approach in our system, as there seemed to be conflicting activities present in the plant extracts that were not present in published bacterial work. Neither GC-MS nor HPLC analysis were able to detect the VOCs, if time had allowed these experiments would have been attempted over a much longer time period. From the current data, it is impossible to definitively conclude whether no acetoin and/or 2,3-butandiol are being produced by the corresponding transgenic lines, whether the volatiles are indeed

produced but are used or broken down immediately, or that these methods need further optimization to detect possible minute levels. Although acetoin and/or 2,3-butanediol were not detected by any of the above-mentioned systems, it seems likely that the VOCs are produced as a significant increase in growth was observed in some of the transgenic lines under certain conditions, however, it should also be kept in mind that the increases in growth could also possibly be due to positional effects from transgene insertion.

4. Conclusions

This study was undertaken to determine the viability of producing acetoin and/or 2,3-butanediol in *Arabidopsis* by using acetolactate in the chloroplast as the precursor in order to enhance plant growth and biomass accumulation. After successful construction of pCambia2300::*FNR:ALDC* and pCambia1300::*FNR:BDH1* constructs, *A. thaliana* was transformed with an *Agrobacterium*-mediated floral dip method. Transgenic plants were generated on selection medium and tested for the incorporation and expression of the *ALDC* and/or *BDH1* genes responsible for the production of acetoin and/or 2,3-butanediol, respectively, *in planta*. A total of 9 lines were generated for each of the single and double transformants. Only two high expressing T₃ generation lines of each single transgene (A3, A6, B2, B6), as well as two T₂ generation double transgenic lines (AB8, AB9) were used for experiments. Various tissue culture experiments were conducted with synthetic VOCs, T₂ generation double transgenic lines and T₃ generation single transgenic lines. Growth experiments in general were difficult to replicate as growth parameters measured (total plant fresh mass, total plant dry mass and total leaf area) between transgenic plants and untransformed control plants gave varying results between different independently-repeated experiments. However, the transgenic *ALDC* lines showed some promising results as they grew significantly larger than the control plants, suggesting that the presence of the *ALDC* transgene allowed the plants to produce acetoin by converting acetolactate. Future analysis could be performed by using better controlled growth conditions for highly reproducible results. Although the growth trails suggested growth promotion by acetoin (and 2,3-butanediol in the double transformants), investigations into the physical presence of acetoin and/or 2,3-butanediol by conducting enzyme assays, GC-MS and HPLC analysis of transgenic plants, gave inconclusive results. The enzymatic assays showed the same trends in oxidation of NADH or colour development between control samples and T₃ *ALDC* transgenic plants and neither GC-MS nor HPLC were able to detect acetoin in the transgenic lines. At this point, it is impossible to conclusively determine whether or not the transgenes are able to effectively generate either of the volatiles *in planta*.

More in-depth analysis of the transgenic lines using cell suspension cultures could possibly result in the detection of acetoin and/or 2,3-butanediol. The genes responsible for acetoin and/or 2,3-butanediol production, *ALDC* and *BDH1* respectively, could also first be analyzed in a bacterial expression system to determine functionality before transforming into plants. To conclude, this study generated some promising results for future studies, with *ALDC* single transgenic lines and an *ALDC/BDH1* double transgenic line showing some significant growth increases, however, it will be crucial to detect the presence of acetoin and/or 2,3-butanediol before further aspects could be investigated.

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